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(54) **Title:** METHODS FOR INHIBITING PROLIFERATION OF ASTROCYTES AND ASTROCYTIC TUMOR CELLS AND FOR ENHANCING SURVIVAL OF NEURONS AND USES THEREOF

(57) **Abstract:** The present invention provides methods for inhibiting proliferation of astrocytes and astrocytic tumor cells. The present invention further provides methods for treating a condition associated with a defect in astrocyte proliferation in a subject, and methods for treating a condition associated with astrocytic tumor cell proliferation in a subject. Additionally, the present invention discloses methods for enhancing survival of neurons. The present invention still further provides methods for treating neural degeneration in a subject. The present invention is also directed to pharmaceutical compositions, comprising NrS1 protein or nucleic acid and a pharmaceutically-acceptable carrier. Finally, the present invention provides a purified NrS1 protein, an agent that binds to the NrS1 protein, a kit comprising an agent that binds to the NrS1 protein, and a composition comprising the NrS1 protein and a carrier.

METHODS FOR INHIBITING PROLIFERATION OF ASTROCYTES AND ASTROCYTIC TUMOR CELLS AND FOR ENHANCING SURVIVAL OF NEURONS AND USES THEREOF

Background of the Invention

[0001] This year, and each year in the foreseeable future, 17,000 people in the United States will be diagnosed with brain tumors. The majority of these tumors will be of astrocyte lineage, and most people diagnosed with these malignancies will die of their diseases. Brain tumors, or intracranial neoplasms, are found in about 2% of all routine autopsies. They are most common in early or middle adult life, but may occur at any age. Their frequency also appears to be increasing in the elderly (30).

[0002] Brain tumors invade and destroy normal tissue, producing such effects as impaired sensorimotor and cognitive function, increased intracranial pressure, cerebral edema, and compression of brain tissue, cranial nerves, and cerebral vessels (30). Metastases may involve the skull or any intracranial structure. The size, location, rate of growth, and histologic grade of malignancy determine the seriousness of brain tumors. Nonmalignant tumors grow slowly, with few mitoses, no necrosis, and no vascular proliferation. Malignant tumors grow more rapidly, and invade other tissues. However, they rarely spread beyond the central nervous system (CNS), because they cause death by local growth. Drowsiness, lethargy, obtuseness, personality changes, disordered conduct, and impaired mental faculties are the initial symptoms in 25% of patients with malignant brain tumors (30).

[0003] While brain tumors, or intracranial neoplasms, are common, they are frequently misdiagnosed (30). Brain tumors may be classified by site (*e.g.*, brain stem, cerebellum, cerebrum, cranial nerves, ependyma, meninges, neuroglia, pineal region, pituitary gland, and skull) or by histologic type (*e.g.*, meningioma, primary CNS lymphoma, or astrocytoma) (30). Common primary childhood tumors are cerebellar astrocytomas and medulloblastomas, ependymomas, gliomas of the brain stem, and congenital tumors. In adults, primary tumors include meningiomas, schwannomas, and gliomas of the cerebral hemispheres (particularly the malignant glioblastoma multiforme and anaplastic astrocytoma, and the more benign astrocytoma and oligodendroglioma). Overall incidence of intracranial neoplasms is essentially equal in males and females, but cerebellar medulloblastoma and glioblastoma multiforme are more common in males (30).

[0004] Gliomas are tumors composed of tissue representing neuroglia in any one of its stages of development (30). They account for 45% of intracranial tumors. Gliomas can encompass all of the primary intrinsic neoplasms of the brain and spinal cord, including astrocytomas, ependymomas, and neurocytomas. Astrocytomas are tumors composed of transformed astrocytes, or astrocytic tumor cells. Such tumors have been classified in order of increasing malignancy: Grade I consists of fibrillary or protoplasmic astrocytes; Grade II is an astroblastoma, consisting of cells with abundant cytoplasm and two or three nuclei; and Grades III and IV are forms of glioblastoma multiforme, a rapidly growing tumor that is usually confined to the cerebral hemispheres and composed of a mixture of astrocytes, spongioblasts, astroblasts, and other astrocytic tumor cells. Astrocytoma, a primary CNS tumor, is frequently found in the brain stem, cerebellum, and cerebrum. Anaplastic astrocytoma and glioblastoma multiforme are commonly located in the cerebrum (30).

[0005] Treatment of brain tumors is often multimodal, and depends on pathology and location of the tumors (30). For malignant gliomas, multimodal therapy, including chemotherapy, radiation therapy, and surgery, is used to try to reduce tumor mass. Regardless of approach, however, prognosis for patients suffering from these tumors is guarded: the median term of survival after chemotherapy, radiation therapy, and surgery is only about 1 year, and only 25% of these patients survive for 2 years. In view of the foregoing, it is imperative that new ways be developed for diagnosing, detecting, and treating malignant gliomas (30).

[0006] Astrocytes also have been implicated in pathologies produced by virtually all neural traumas, including CNS injury and neuronal cell death resulting from neurodegenerative disease. In the case of CNS injury, for example, resulting astrocytosis is thought to be a major contributor to the formation of a glial scar, which is believed to present a major barrier to productive neural regeneration (6). Therefore, a primary goal in the design of therapeutics for both CNS trauma and neurodegenerative diseases is the elucidation of mechanisms for limiting glial scar formation.

[0007] Head injuries cause more deaths and disability than any other neurologic condition before age 50, and occur in more than 70% of accidents – the leading cause of death in men and boys less than 35 years of age. Mortality from severe injury approaches 50%, and is only modestly reduced by treatment. Damage may result from skull penetration or from rapid brain acceleration or deceleration, resulting in injury to surrounding tissue. Currently, there is no treatment for astrocytosis resulting from head trauma.

[0008] Alzheimer's disease is a neurodegenerative disease characterized by a progressive, inexorable loss of cognitive function (30). The pathogenesis of Alzheimer's disease is associated with an excessive number of neuritic, or senile, plaques (composed of neurites, astrocytes, and glial cells around an amyloid core) in the cerebral cortex, and neurofibrillary tangles (composed of paired helical filaments). Approximately 4 million Americans suffer from Alzheimer's disease, at an annual cost of about $90 billion. The disease is about twice as common in women as in men, and accounts for more than 65% of the dementias in the elderly. While senile plaques and neurofibrillary tangles occur with normal aging, they are much more prevalent in persons with Alzheimer's disease. To date, a cure for Alzheimer's disease is not available, and cognitive decline is inevitable.

[0009] At present, there are no specific treatments for astrocytosis. In addition, while there are standard chemotherapeutic, radiotherapeutic, and surgical treatments for astrocytoma, these therapies are fraught with severe limitations, and are often palliative rather than curative. Accordingly, there is a great need to develop methods of treating astrocytomas, astrocytosis, and other conditions associated with a proliferation of astrocytes or astrocytic tumor cells. An understanding of the basic biology of neuron-glial interaction may provide insight into the elucidation of such treatment options.

Summary of the Invention

[0010] The present invention is based upon the discovery that CD81 on the surface of astrocytes modulates proliferation of astrocytes in neural tissue, and is not expressed in astrocytic tumor cells, and the discovery that NrS1 on the surface of neurons is involved in bi-directional signaling to astrocytes and neurons following contact with CD81. On the basis of these findings, the present invention provides methods for enhancing survival of neurons. The methods comprise contacting the neurons with CD81 protein or a CD81 derivative, in an amount effective to enhance survival of the neurons.

[0011] The present invention is also directed to methods for treating neural degeneration in a subject in need of treatment. The methods comprise contacting neurons in the subject with CD81 protein or CD81 derivative in an amount effective to enhance survival of neurons, thereby treating the neural degeneration.

[0012] The invention is additionally directed to methods for treating neural degeneration in a subject in need of treatment. The methods comprise activating NrS1 in the subject.

[0013] In other embodiments, the present invention is directed to methods for inhibiting proliferation of astrocytes. The methods comprise contacting the astrocytes with an amount of NrS1 protein or NrS1 derivative effective to inhibit proliferation of the astrocytes.

[0014] Additionally, the invention is directed to methods for treating a condition associated with a defect in astrocyte proliferation in a subject in need of treatment. The methods comprise contacting astrocytes in the subject with NrS1 protein or NrS1 derivative in an amount effective to inhibit proliferation of the astrocytes, thereby treating the condition associated with a defect in astrocyte proliferation.

[0015] The present invention is additionally directed to methods for treating a condition associated with a defect in astrocyte proliferation in a subject in need of treatment. The methods comprise activating CD81 in the subject.

[0016] In additional embodiments, the invention is also directed to pharmaceutical compositions. The compositions comprise NrS1 and a pharmaceutically-acceptable carrier. Other compositions of the invention comprise nucleic acids encoding NrS1 and a pharmaceutically-acceptable carrier.

[0017] In further embodiments, the invention is directed to methods of determining whether a subject has an astrocytoma. The methods comprise assaying for CD81 expression in a diagnostic sample of cells of astrocytic lineage of the subject, where no detection of expression of CD81 in the cells of astrocytic lineage is diagnostic of an astrocytoma.

[0018] Finally, the present invention provides purified NrS1 protein, agents that bind to the NrS1 protein, kits comprising an agent that binds to the NrS1 protein, and compositions comprising the NrS1 protein and a carrier.

[0019] Additional objects of the present invention will be apparent in view of the description which follows.

Brief Description of the Drawings

[0020] FIGS. 1A is a western blot, and FIGS. 1B and 1C are fluorescence micrographs, that demonstrate that CD81 is expressed on the surface of the astrocyte. After the expression of CD81 message was identified by differential screen, protein expression was determined by Western blot analysis. (A) While astrocytes express ample CD81, the C6 glioma cell line is CD81-negative. To localize the protein expression in the astrocyte, astrocytes were cultured either alone (B) or in the presence of neurons (C) for 48 h, then fixed and stained for the

expression of CD81 on the cell surface, using the monoclonal antibody 2F7. The arrows in (B) point out the expression of CD81 along the astrocyte processes – a domain of the cell critical for neuronal interaction (10).

[0021] FIGS. 2A-2H are graphs and micrographs establishing that CD81 is a critical mediator of neuron-astrocyte interactions, by showing that anti-CD81 antibody Eat1 effectively interferes with normal neuron-mediated astrocyte arrest. (A) In the presence of increasing concentrations of Eat1 monoclonal antibody (mAB), there was a loss of neuron-mediated astrocyte proliferative arrest (closed bars); the antibody had no effect on astrocyte proliferation in the absence of neurons (open bars). (B) In contrast, 2F7 augmented neuronally-induced astrocyte proliferative arrest, such that, in the presence of this mAb and neurons, astrocyte proliferation was further reduced over the level seen with neurons alone. (C) Immunofluorescence studies of astrocytes stained with anti-GFAP antiserum in neuron-astrocyte co-cultures, in the presence of Eat1, showed a dependence on the Eat1 epitope for normal responsiveness to neuronally-induced, astrocyte process formation. In contrast, blocking the Eat2 epitope had no effect on astrocyte responsiveness to neurons, as evidenced by the complex GFAP processes seen in these cultures (D) which had the same appearance as control co-cultures (E). While mAbs Eat1 and 2F7 had profound effects on astrocytic responses to neurons, they had no observable effects on neuronal survival or axonogenesis. Neuron-astrocyte co-cultures were also stained with the mAb TuJ1, which recognizes a neuron-specific βIII tubulin isoform (3). The extent and quality of neurites were comparable in the presence of Eat1 (F) and 2F7 (G), and in control co-cultures (H).

[0022] FIGS. 3A-3C are fluorescence micrographs demonstrating that GST-CD81 binds to neurons, not astrocytes. Highly-enriched cultures of either neurons or astrocytes from P4 rat cerebellum were established, as previously described (28). The cells, which were plated at equivalent densities, were cooled on ice to prevent internalization, then incubated with 10 μg/ml of bacterially-expressed GST-CD81 for 1 h. The cells were fixed, and stained with a goat anti-GST antibody, followed by an Alexa red conjugated rabbit anti-goat secondary. FIG. 3A shows the absence of background staining (no primary antibody control). FIG. 3B shows the binding of the CD81 fusion protein to the surface of the neuron. In contrast, only the few contaminating neurons in the astrocyte-enriched fraction bound the fusion protein (C).

[0023] FIG. 4 is a graph that illustrates that soluble CD81 competes with astrocyte-expressed CD81, and blocks neuron-induced astrocyte quiescence. Increasing concentrations of soluble GST-CD81 were added to co-cultures of neurons and astrocytes. The GST-CD81

competed for neurons with the expressed CD81, thereby blocking neuronal CD81-receptor binding at the astrocyte cell surface. As a result of this competition, astrocytes remained in the cell cycle. 40-50% of neuron-induced inhibition of astrocyte proliferation was achieved with as little as 1 μg/ml of GST-CD81. Maximal inhibition was obtained with 3 μg/ml of soluble protein. The soluble proteins had no observable effects on astrocyte proliferation in the absence of neurons. The specificity of the effect of GST-CD81 was verified by the addition of another, irrelevant GST fusion protein, GST-SCIP, which had no effect on neuron-induced astrocyte quiescence at any concentration tested. Statistical analysis was carried out using the two-tailed students' t-test.

[0024] FIG. 5 is a pair of graphs that shows that CD81 is required for neuron-induced astrocyte growth regulation. Mixed cultures of wild-type, CD81+/-, or CD81-/- cerebellar astrocytes and granule cell neurons were established and evaluated for the role of endogenous CD81 in neuron-induced astrocyte responses. Astrocyte proliferation was determined by double labeling for GFAP and BrdU 48 h after the cultures were established, as described below. Astrocyte proliferation in wild-type co-cultures was determined, and arbitrarily set at 1. CD81 haplo-insufficient astrocytes showed a slight loss of neuron responsiveness (20%). However, astrocytes null at the CD81 locus lost all responsiveness to neurons under these conditions, doubling in number within 48 h after explantation. Assays were done in triplicate for each animal tested, and the overall experiment was repeated three times.

[0025] FIG. 6 is a northern blot illustrating that CD81 RNA is absent from a range of astrocytic tumor cell lines. A hallmark of cell transformation is a loss of proliferative arrest in response to naturally-occurring cues. To determine if astrocytic tumor cell lines expressed altered levels of CD81, the inventor extracted RNA from a variety of cell lines, and performed northern blot analysis. Not unexpectedly, CD81 message was found in wild-type astrocytes. The CD81 levels increased by approximately two-fold when the cells were co-cultured for 48 h with neuronal membranes, suggesting a positive feed-back mechanism affecting CD81 expression. In stark contrast, none of the tumor cell lines tested had any detectable CD81 message, even when the blot was over-exposed (not shown). These astrocytic tumors tested were rat: C6 and 9L; human: A172 and U251MG; and mouse: LN308 and LN18. An 18S probe was used as a loading control for RNA.

[0026] FIG. 7 depicts the nucleotide sequence of human CD81.

[0027] FIG. 8 depicts the amino acid sequence of human CD81.

[0028] FIG. 9 is a graph that demonstrates the saturable nature of neuronal protein binding to astrocytes. Cerebellar granule cell neuron proteins (closed diamonds) or NGF-differentiated PC12 cell membrane proteins (open diamonds) were isolated, as described below, and allowed to bind to monolayers of astrocytes. The CNS neuron proteins saturated binding at ~10 μg/10^5 astrocytes; in contrast, the PC12 cell membranes did not bind to the astrocytes at all.

[0029] FIGS. 10A is a silver stained gel and 10B shows micrographs that illustrate column chromatographic purification of proteins active in neuron-induced astrocyte growth arrest. (A) The inventor established a purification scheme in which lipophilic neuronal proteins were isolated (lane 1), then passed over an FPLC anion exchange column. The peak between 240 mM NaCl and 260 mM NaCl was collected (lane 2), dialyzed, and passed over a column of total astrocyte membrane proteins. This column then was eluted with 10 mM EDTA (lane 3), followed by 500 mM NaCl (lane 4). All of the antiproliferative activity was contained in lane 4 (see Table 1). The proteins were run on a 6% SDS-PAGE gel, and imaged by silver staining. The arrow points to the major enriched band at ~65 kD. (B) The protein from lane 4 (panels a and b), or BSA (panels c and d), was coupled to a nitrocellulose-coated tissue culture surface, and non-specific binding was quenched with BSA. Astrocytes were added to a 48-h culture, and the cells then were stained with rabbit anti-GFAP (panels a and c) and mAb anti-statin (panels b and d). These representative fields demonstrate that statin translocation to the nucleus – a marker of cells in G_0 – occurs specifically when the cells are exposed to proteins that were NaCl-eluted from the final column.

[0030] FIG. 11 is a graph that illustrates that GM1109 competes for astrocyte-expressed CD81, and blocks neuron-induced astrocyte quiescence. Upon addition of increasing concentrations of GM1109 to co-cultures of neurons and astrocytes, GM1109 competed for astrocyte-expressed CD81, thereby blocking the binding of the neuronal CD81-receptor to the astrocyte cell surface. As a result of the competition, astrocytes remained in the cell cycle. 40-50% of neuron-induced inhibition of astrocyte proliferation was achieved with as little as 1 μg/ml of GM1109; maximal inhibition was obtained with 3 μg/ml of soluble protein. The soluble proteins had no observed effect on astrocyte proliferation in the absence of neurons. Compare the open bars corresponding to control conditions with those corresponding to conditions in which protein was added. The specificity of the GM1109 effects was verified by the addition of an irrelevant drug, GM1110, which had no effect on neuron-induced astrocyte quiescence at any concentration tested. Statistical analysis was carried out using the two-tailed students' *t*-test.

[0031] FIG. 12 shows micrographs demonstrating that GM1109 binds to neurons, but not to astrocytes. Highly enriched cultures of either neurons or astrocytes from P4 rat cerebellum were established, as described below. The cells, which were plated at equivalent densities, were cooled on ice, to prevent internalization, and incubated with 10 µg/ml of bacterially-expressed GM1109 for 1 h. The cells were fixed, then stained with a goat anti-GST antibody, followed by an Alexa red conjugated rabbit anti-goat secondary antibody. Panel a shows the absence of background staining (no primary antibody control). Panel b shows the binding of GM1109 to the surface of the neuron. In contrast, only a few contaminating neurons in the astrocyte-enriched fraction bound the fusion protein (panel c).

[0032] FIGS. 13A and 13B are blots demonstrating that GM1109 specifically binds to neuronal protein extract. Far-Western analysis using soluble GM1109 showed that this protein specifically binds to a neuronal, detergent-soluble protein of approximately 70 kD. Granule cell neurons (GCN) from either rat or mouse, as well as rat Schwann cells and 3T3 cells, were solublized in Triton X-100. Ten micrograms (10 µg) of each protein were separated on a 12% SDS-PAGE gel. In addition, 1 µg of the probe also was run on the gel, and was then transferred to nitrocellulose. The blot on the left (A) was probed with GM1109, and the blot on the right (B) was probed with GST alone. Both were probed at 4°C, and thoroughly washed. The GST or GM1109 was visualized with an anti-GST antibody. GM1109 reacted solely with a single band in the neuronal fractions, thereby demonstrating the specificity of GM1109 interaction. The GM1109-positive control was recognized by the anti-GST secondary antibody in both panels, and served as an internal control of the specificity of GM1109 and the secondary antibody.

[0033] FIG. 14 is a silver stained gel that shows that GM1109 pulls down NrS1 from neurons. The inventor purified rat cerebellar granule cell neurons, as previously described. After contaminating astrocytes were preferentially adhered out, the cells were mixed with 10 µg of soluble GM1109. The cells were lysed, and the GM1109 and associated proteins were pulled down with Sepharose-glutathione beads (lane 1). 3T3 cells were treated identically after the purification step. The pull-down from the 3T3 cells is shown in lane 2. The proteins were separated on a 10% SDS-PAGE gel, which was then silver stained. The heavy bands at ~30 kD represent the starting GM1109, which was purified from bacterial lysate by Sepharose-glutathione chromatography. The single band at 70 kD represents the lone protein species expressed by the neuron that binds to astrocyte-expressed GM9 in *trans*.

[0034] FIG. 15 is a western blot that illustrates that GM1109 induces phosphorylation of NrS1. Human granule cell neurons were purified from a 22-week old male fetal cerebellum, as described below. Following isolation, the cells were cultured in serum-free medium for 1 h and harvested. GM1109 then was added for the indicated times and the cells were lysed. The GM1109 and associated NrS1 proteins were pulled down with glutathione-conjugated Sepharose (Pharmacia), separated on a 10% SDS-PAGE, transblotted to a nitrocellulose membrane, and probed with a polyclonal anti-phosphotyrosine antiserum. The data show that NrS1 is phosphorylated, in response to CD81 signaling, in a time-dependent manner - a result consistent with active signaling from the astrocyte to the neuron, *via* a CD81-NrS1 pathway. αY^* = phosphotyrosine

[0035] FIGS. 16A-16C are micrographs that demonstrate that full astrocyte differentiation depends upon NrS1 and other neuronal signals. Astrocytes were grown in the presence or absence of neurons, with or without dbcAMP. (A) Astrocytes in the presence of complete medium supplemented with dbcAMP grew to confluence, with no sign of astrocytic-process outgrowth. (B) When the same cells were cultured with NrS1, they growth-arrested, but failed to form processes. (C) However, when the astrocytes were provided with both NrS1 and dbcAMP, they both growth-arrested, and formed complex processes, mimicking astrocyte responses to viable neurons.

[0036] FIGS. 17A-17C are micrographs, and FIG. 17D is a western blot showing that the astrocyte mimetic, GM1109, induces the rapid cleavage of neuronal, transmembrane neuregulin (Nrg), and the migration of ICD_{Nrg} to the neuronal nucleus. Cerebellar granule cells were acutely dissociated from P3 Long-Evans rats, and purified, as previously described (27). The cells were washed free of serum, then plated in defined medium that was supplemented with 10% BSA. After the cells were allowed to adhere to the coverslips, either control GST protein (A) or GM1109 (B) was added to the medium, and the cells were allowed to incubate for 5 min. The cells were fixed, permeabilized, and stained with an antibody that recognizes the intracellular domain of neuregulin (ICD_{Nrg}). As the figure illustrates, the binding of GM1109 to the neuron induces nuclear translocation of the ICD_{Nrg}. As a result of GM1109, the Nrg extracellular domain (ECD) is rapidly shed to the supernatant. (D) Treatment of neurons with GM1109 caused a 2-fold increase in the amount of soluble Nrg in the supernatant, within 5 min, as compared to neurons treated with an irrelevant peptide.

[0037] FIG. 18 is a micrograph showing that GM1109 induces neuronal survival and neuritogenesis. Neonatal rodent cerebellar neurons were purified to >99% homogeneity, and cultured at low density, in the presence of either a control peptide or GM1109, for 96 h. The cells were fixed and stained with anti-GFAP and anti-neuron-specific tubulin antibodies. Treatment with the CD81/astrocyte mimetic, GM1109, induced neuronal survival and robust axonal outgrowth (panels B and C, at the right). In contrast, most cells grown in the absence of GM1109 failed to survive; those that did survive extended meager processes (panel A, at the left).

[0038] FIGS. 19A and 19B are illustrations depicting a model of the molecular basis of neuron-astrocyte interactions. (A) Astrocytes express CD81, which is required for neuron-induced growth arrest. Neurons signal to astrocytes by binding to CD81 via NrS1, resulting in the rapid phosphorylation of NrS1. Concomitantly with these events, neuronally-expressed, transmembrane Nrg is cleaved, resulting in the secretion of Nrg. Secreted Nrg binds the astrocyte erbB2/4 heterodimers, inducing elevation of intracellular cAMP in the astrocyte. Together with CD81 signaling, these events lead to astrocyte growth arrest and maturation. In addition, the cleavage of neuronal Nrg results in the translocation of ICD_{Nrg} to the nucleus, as well as neuronal survival and differentiation. (B) GM1109 acts as an astrocyte mimetic on the neuron. In the absence of added growth factors or astrocytes, treatment of neurons with GM1109 results in cleavage of Nrg, translocation of ICD_{Nrg} to the neuronal nucleus, and neuronal survival.

[0039] FIGS. 20A is a graph and 20B shows micrographs demonstrating that GM1416 rescues CNS neurons from cell death. (A) Neonatal rodent cerebellar neurons were purified to >99% homogeneity, and cultured at low density in the presence of 10 μg/ml GM1109 as a positive control, or 1 μg/ml, 3 μg/ml, and 10 μg/ml of the mimetics GM1414, GM1415, and GM1416, respectively, or no treatment. The peptides were added fresh daily for 5 days, and then viability was assayed using a commercial live/dead assay from Molecular Probes. As expected, most neurons died in the absence of treatment. However, consistent with data shown in FIG. 19, GM1109 maintained neuronal viability. At the lowest concentration of GM1414, neuronal survival was almost at GM1109 levels; however, at increasing concentrations, neuronal survival was lost. GM1415 had no neuronal-survival-inducing activity. In contrast, GM1416 potently induced neuronal survival, surpassing even that observed for GM1109. (B) Neurons treated with GM1416 survived, and produced extensive neurites. As a positive control, purified granule cell neurons were treated with 10 μg/ml of GM1109, inducing survival and neuritogenesis (panel a). Sister cultures were treated with GM1416, a small peptide derivative of GM1109, which also

induced survival and neurite production (panel b). In contrast, neurons cultured in the absence of these molecules promptly died, as determined by inclusion of the EtBr stain (panel c).

[0040] FIG. 21 shows micrographs establishing that removal of CD81 by homologous recombination results in global astrocytosis. The vast majority of CD81-/- animals die perinatally. However, a small number of animals live for ~2 weeks. Examination of the brains of these outlying animals shows a global astrocytosis. Sections were cut through the cerebellum of either wild-type (+/+), or CD81-/- (-/-) animals and stained with an antibody to GFAP or for NrS1 expression. There are a small number of GFAP-positive astrocytes in the wild-type cerebellum, and those cells that are present are tightly apposed to the Purkinge (PC) and granule cell neuron soma. In the molecular layer (ML), astrocyte are running perpendicular to the granule cell processes in the +/+ cerebellum. In contrast, the -/- cerebellum shows a large increase in astrocyte cell number, Bergmann-type glia (arrows) remaining in the ML, loss of PCs (asterisks), and a loss of the tight contacts between the neurons and astrocytes (arrow heads).

[0041] FIG. 22 shows micrographs of CD81-/- animals suffering neuronal atrophy and death, as well as heterotopia. Cerebelli of two week old CD81 +/+ and -/- were stained for NrS1 expression. In the absence of CD81, Purkinje cells (adjacent to the dotted lines in both panels) undergo severe atrophy and death. In addition, there is heterotopia, in which some granule cell neurons failed to complete their migration along Bergmann glia into the granule cell layer (GCL). This tissue is outlined in dark dashes.

[0042] FIG. 23 is a micrograph showing that CD81 expression is lost in astrocytomas in situ. We have stained numerous biopsy and resection specimens from patients with confirmed diagnoses of astrocytoma, all of which have lost CD81 expression in the neoplastic tissue. This representative needle biopsy is from a 34 year old woman who died of her disease. The tissue was stained with an antibody, 2F7, which stained the astrocytes in the margins around the mass, but failed to stain the astrocytic cells that comprise the bulk of the tumor. The box in the lower left is an enlargement of CD81 positive cells at the border of the lesion.

[0043] FIG. 24 shows micrographs providing evidence that CD81 is required for initial astrocyte/neuron interactions. In the absence of neurons, acutely dissociated astrocytes undergo rapid extensions and retractions of membrane blebs (a). This behavior lasts from hours to days. When CNS neurons are added to these cells, the astrocytes rapidly stop blebbing upon neuronal contact, when they exit the cell-cycle and extend organized processes (b). Astrocytoma cells (C6 in this case) bleb in a manner similar to astrocytes (c), however, these cells ignore the addition of

neurons (d). When the astrocytoma cells are transfected with CD81, they regain responsiveness to neurons (e). (A=astrocyte; Am=astrocytoma; N=neuron).

[0044] FIG. 25 shows micrographs providing evidence that re-expression of CD81 rescues astrocytoma neuron responsiveness. C6 astrocytoma cells were stably transfected with a CD81 cDNA and cloned by limiting dilution. The transfectants (a) or the parent cell line (b) showed no difference in growth or morphology in the absence of neurons. However, when neurons were added to the CD81 expressing cells (c), they exited the cell-cycle, extended processes and remained quiescent as long as the neurons were present. In contrast, the parental C6 cells were insensitive to the neuronal anti-proliferative signal, and continued to expand (d). The arrow heads point to the remaining neurons in panel d.

[0045] FIG. 26 shows two-dimensional gel electrophoresis separation of NrS1 pulled down by GM1109. NrS1 was pulled down, as described in the body of the application. This material was separated in the first dimension using iso-electric focusing, and in the second dimension based on molecular mass. The four predominant spots shown were subjected to mass spectroscopy analysis and Edman degradation analysis. These analyses yielded data consistent with our previous observations that NrS1 is a tyrosine kinase. Moreover they suggested that NrS1 has been previously identified as a orphan receptor tyrosine kinase termed EHK-1. EHK-1 is a member of the ephrin family of tyrosine kinases.

[0046] FIG. 27 shows the published nucleotide sequence of EHK-1. GenBank Accession number XM_046083.

[0047] FIG. 28 shows the published amino acid sequence of EHK-1. GenBank Accession number XP_046083.2.

[0048] FIG. 29 shows the sequence of a novel exon 5' to the published sequence of EHK-1.

[0049] FIG. 30 shows micrographs establishing that NrS1 is expressed by the majority, if not all, cortical, hippocampal, thalamic, motor, and cerebellar neurons. Our biochemical data demonstrated that the CD81 mimetic, GM1109, bound to and was used to identify NrS1. Based on the ability of GM1109 to bind to this ligand, we used biotinylated GM1109 to localize NrS1 expression. Binding of this probe to mouse brain shows NrS1 is expressed by virtually all cortical, hippocampal and thalamic neurons. Biotinylated GST was used as a control, which failed to bind to the brain sections.

[0050] FIG. 31 shows micrographs establishing that NrS1 is expressed by most retinal neurons. Staining of rat retinae with the same probe used in FIG. 30 revealed NrS1 expression by ganglion cell neurons, photo receptors and most cells in the inner nuclear layer (A). Retinae were unstained by the control, biotinylated GST probe (B).

[0051] FIG. 32 shows micrographs establishing that NrS1 is expressed by enteric neurons. Staining of mouse intestine with the same probe used in FIG. 30 revealed NrS1 expression by the myenteic plexus (mp) and the submucosal plexus (sp), the plexi of the gut. In addition, the sympathtic nerves (sn) that innervate the intestinal wall (iw) are also strongly NrS1 positive. GM1109 was visualized with either a colorometric substrate (b and d), or a fluorescinated streptavidan substrate (a and c).

[0052] FIG. 33 is an illustration of a model of GM1109/NrS1 interactions. GM1109 is a CD81 mimetic, and binds to NrS1, as shown herein. Binding of GM1109 to its ligand induces the rapid phosphorylation of NrS1.

[0053] FIG. 34 is a western blot showing that binding of CD81 and CD81 mimetics to NrS1 induces shedding of the extracellular domain of Nrg to the medium. One minute following binding of GM1109 to neurons, the medium was collected. Fifty μl of the medium was electrophoresed on a 12% SDS-PAGE gel, then transferred to nitrocellulose. The membrane was probed with an anti-Nrg antibody, specific for the mature, secreted form of the growth factor. As shown, there is a rapid accumulation of the protein in the medium, while there is a concomitant translocation of the intracellular domain of the protein to the neuronal nucleus (see above).

[0054] FIG. 35 shows western blots establishing that GM1109-induced, neuron-secreted Nrg induces a rapid phosphorylation of its receptor, ErbB2, on the astrocyte. The medium used in FIG. 34 was added to astrocytes that had been cultured for 48 hours in defined medium. The cells were lysed at the indicated times, separated by 8% SDS-PAGE, and transferred to nitrocellulose. The membrane was probed with a mAb specific for the phosphorylated form of ErbB2, part of the Nrg receptor heterodimer. The blot shows full phosphorylation of the receptor within 5 minutes of the addition of the medium.

[0055] FIG. 36 shows micrographs demonstrating that GM1416 rescues retinal ganglion cells from death in a pressure induced stroke model. Ten micrograms of GM1416RI, a retro-inverso stabilized form of GM1416, was infused into the posterior chamber of the eye at the time of stroke. Animals were sacrificed at the indicated times, and the retinae processed for NrS1

expression. The ganglion cell soma are preserved, as are their axons in the inner plexiform layer (IPL).

[0056] FIG. 37 is a graph showing preservation of axon tracks is a surrogate marker for neuronal viability. The thickness of the IPL was measured in control and GM1416RI treated retinae. GM1416RI preserves the full thickness of the IPL at 72 hours after stroke.

[0057] FIG. 38 provides sequences of active fragments of CD81. A - GM1109; B - GM1414; C - GM1415; D - GM1416; E - active 15-mer of GM1416.

Detailed Description of the Invention

[0058] The present invention provides methods for inhibiting proliferation of astrocytes, by contacting astrocytes with an amount of CD81 effective to inhibit proliferation of astrocytes. Unless otherwise indicated, "CD81" includes any mammalian a CD81 protein (p27), a CD81 analogue, and a CD81 fragment. As used herein, one form of CD81 protein has the amino acid sequence set forth in FIG. 8. The skilled artisan would expect that any mammalian CD81 has at least 80% amino acid homology to any other mammalian CD81. It would also be expected that any mammalian CD81 would be functional in the methods of the invention in any mammal. However, it is preferred that the CD81 utilized in those methods be from the same species, to avoid possible immune rejection problems.

[0059] A "CD81 analogue", as defined herein, is a functional variant of a CD81 protein, having CD81-protein biological activity, that has at least 60%, preferably at least 70%, more preferably at least 80%, even more preferably at least 90%, still more preferably at least 95%, and most preferably at least 97% amino acid sequence homology with the CD81 protein, as well as a fragment of the CD81 protein having CD81-protein biological activity, such as GM1109, GM1416, and the active 15-mer from GM1416 (FIG. 38). The skilled artisan could envision many variants or fragments of CD81 that would be expected to have CD81-protein biological activity. As used herein, the term "CD81-protein biological activity" refers to protein activity which modulates and inhibits proliferation of astrocytes and astrocytoma cells, as disclosed herein. Additionally, as used herein, a "CD81 derivative" is a chemical substance derive from CD81, either directly or by modification, truncation, or partial substitution. For example, the CD81 derivative for use in the present invention may be the extracellular domain (ECD) of CD81. In addition, the CD81 derivative of the present invention may be retro-inverso versions of CD81 or

the variants or fragments, in which the primary sequence of the peptides is in an inverse orientation and comprise D-amino acids. These modifications are known to stabilize the protein.

[0060] CD81 and its analogues and derivatives may be produced synthetically or recombinantly, or may be isolated from native cells; however, they are preferably produced synthetically, using conventional techniques and cDNA encoding CD81 (FIG. 7). In one embodiment of the present invention, the astrocytes are undifferentiated, *i.e.*, they are not in cell-cycle arrest, and they have not formed complex processes.

[0061] The methods of the present invention may be used to inhibit proliferation of astrocytes *in vitro*, or *in vivo* in a subject. As used herein, the term "inhibit proliferation of astrocytes" means inhibit cell division and growth of astrocytes, and includes limiting the proliferative rate of astrocytes, as disclosed herein. Inhibition of the growth and proliferation of astrocytes may be detected by known procedures, including any of the methods, molecular procedures, and assays disclosed herein.

[0062] In accordance with the methods of the present invention, CD81 may be contacted with astrocytes *in vitro*, or *in vivo* in a subject, by introducing the CD81 protein into the membranes of astrocytes, or by introducing into the astrocytes a nucleic acid encoding CD81 in a manner permitting expression of CD81 protein. The subject may be any animal, but is preferably a mammal (*e.g.*, humans, domestic animals, and commercial animals). More preferably, the subject is a human. The astrocytes may be contained in neural tissue and other tissue of the nervous system of the subject, either alone or with other types of neural cells, including, for example, neurons and oligodendroglia. Astrocytes may be detected in tissue of the subject by standard detection methods readily determined from the known art, examples of which include, without limitation, immunological techniques (*e.g.*, immunohistochemical staining), fluorescence imaging techniques, and microscopic techniques.

[0063] CD81 protein may be introduced into the membranes of astrocytes, either *in vitro* or *in vivo* in a subject, by known techniques used for the introduction of proteins into cell membranes (*e.g.*, by means of micro-encapsulated preparations, such as liposomes). The amount of CD81 protein to be used is an amount effective to inhibit proliferation of astrocytes, as defined above, and may be readily determined by the skilled artisan.

[0064] For introduction of CD81 protein by way of liposome delivery, liposomal vesicles may be prepared by various methods known in the art, and liposome compositions may be prepared using any one of a variety of conventional techniques for liposome preparation known to

those skilled in the art. Examples of such methods and techniques include, without limitation, chelate dialysis, extrusion (with or without freeze-thaw), French press, homogenization, microemulsification, reverse phase evaporation, simple freeze-thaw, solvent dialysis, solvent infusion, solvent vaporization, sonication, and spontaneous formation. Preparation of the liposomes may be carried out in a solution, such as an aqueous saline solution, aqueous phosphate buffer solution, or sterile water. Liposome compositions also may be prepared by various processes involving shaking or vortexing. CD81 protein may be incorporated into the layers of a liposome such that its intracellular domain extends outside the liposome, and its extracellular domain extends into the interior of the liposome. The liposome containing CD81 then may be fused with an astrocyte, in accordance with known methods of fusion of liposomes to cell membranes, such that the CD81 protein is delivered into the membrane of the astrocyte with its intracellular domain extending into the interior of the astrocyte, and its extracellular domain extending outside the membrane of the astrocyte.

[0065] In the methods of the present invention, CD81 also may be contacted with astrocytes, either *in vitro* or *in vivo* in a subject, by introducing into a sufficient number of astrocytes of the subject a nucleic acid encoding CD81, in a manner permitting expression of CD81. The nucleic acid may be introduced using conventional procedures known in the art, including, without limitation, electroporation, DEAE Dextran transfection, calcium phosphate transfection, monocationic liposome fusion, polycationic liposome fusion, protoplast fusion, creation of an *in vivo* electrical field, DNA-coated microprojectile bombardment, injection with recombinant replication-defective viruses, homologous recombination, *in vivo* gene therapy, *ex vivo* gene therapy, viral vectors, and naked DNA transfer, or any combination thereof. Recombinant viral vectors suitable for gene therapy include, but are not limited to, vectors derived from the genomes of viruses such as retrovirus, HSV, adenovirus, adeno-associated virus, Semiliki Forest virus, cytomegalovirus, and vaccinia virus. The amount of nucleic acid encoding CD81 to be used is an amount that will express CD81 protein in an amount effective to inhibit proliferation of astrocytes, as defined above. These amounts may be readily determined by the skilled artisan.

[0066] It is also within the confines of the present invention that a nucleic acid encoding CD81 may be introduced into suitable cells *in vitro*, using conventional procedures, to achieve expression in the cells of CD81 protein. Cells expressing CD81 protein then may be introduced into a subject to inhibit proliferation of astrocytes *in vivo*. In such *ex vivo* gene therapy

approaches, the cells are preferably removed from the subject, subjected to DNA techniques to incorporate nucleic acid encoding CD81, and then reintroduced into the subject.

[0067] The ability of CD81 to modulate astrocyte proliferation renders CD81 particularly useful for treating conditions associated with a defect in astrocyte proliferation. As used herein, "a defect in astrocyte proliferation" includes pathologic proliferation of astrocytes in a particular tissue, as compared with normal proliferation in the same type of tissue. It is believed that, by modulating astrocyte proliferation, CD81 will be useful for the treatment of conditions associated with defects in astrocyte proliferation. It is further believed that CD81 would be effective either alone or in combination with therapeutic agents, such as chemotherapeutic agents or antiviral agents, which are typically used in the treatment of these conditions.

[0068] Accordingly, the present invention provides methods for treating a condition associated with a defect in astrocyte proliferation in a subject in need of treatment, comprising contacting astrocytes in the subject with an amount of CD81 effective to inhibit proliferation of astrocytes, thereby treating the condition. As described above, the subject may be any animal, but is preferably a mammal (*e.g.*, humans, domestic animals, and commercial animals). More preferably, the subject is a human.

[0069] Examples of conditions associated with a defect in astrocyte proliferation include, without limitation, astrocytosis, glial scars, hyperplasia, neoplasia, and neuritic plaques (particularly those commonly found in Alzheimer's disease patients). As used herein, "astrocytosis" refers to the proliferation of astrocytes owing to a destruction of nearby neurons. As further used herein, "hyperplasia" refers to the abnormal multiplication or increase in the number of normal astrocytes, in normal arrangement, within a tissue. In one embodiment of the present invention, the condition associated with a defect in astrocyte proliferation is astrocytosis. In another embodiment of the present invention, the condition associated with a defect in astrocyte proliferation is a neuritic plaque.

[0070] Astrocytosis, glial scars, hyperplasia, neoplasia, neuritic plaques, and other conditions associated with a defect in astrocyte proliferation may be caused by, or associated with, a variety of factors, including, without limitation, neuronal cell death and neural degeneration resulting from neurodegenerative diseases, CNS traumas, and the acquired secondary effects of non-neural dysfunction. Examples of neurodegenerative diseases include, without limitation, Alzheimer's disease, amyotrophic lateral sclerosis (Lou Gehrig's Disease), Binswanger's disease, Huntington's chorea, multiple sclerosis, myasthenia gravis, Parkinson's disease, and Pick's disease.

Examples of CNS traumas include, without limitation, blunt trauma, hypoxia, and invasive trauma. Examples of acquired secondary effects of non-neural dysfunction include, without limitation, cerebral palsy, congenital hydrocephalus, muscular dystrophy, stroke, and vascular dementia.

[0071] In the treatment of a condition associated with a defect in astrocyte proliferation, CD81 may be contacted with astrocytes by introducing the CD81 protein into the membranes of astrocytes, in accordance with known methods (*e.g.*, liposome delivery), as described above. The amount of CD81 protein to be used is an amount effective to inhibit proliferation of astrocytes, as defined above, and may be readily determined by the skilled artisan.

[0072] Alternatively, in accordance with known methods, including those described above, CD81 may be contacted with astrocytes to treat a condition associated with a defect in astrocyte proliferation by introducing into the astrocytes a nucleic acid encoding CD81, in a manner permitting expression of CD81 protein. The nucleic acid may be introduced using conventional procedures known in the art, including, without limitation, electroporation, DEAE Dextran transfection, calcium phosphate transfection, monocationic liposome fusion, polycationic liposome fusion, protoplast fusion, creation of an *in vivo* electrical field, DNA-coated microprojectile bombardment, injection with recombinant replication-defective viruses, homologous recombination, *in vivo* gene therapy, *ex vivo* gene therapy, viral vectors, and naked DNA transfer, or any combination thereof. Recombinant viral vectors suitable for gene therapy include, but are not limited to, vectors derived from the genomes of viruses such as retrovirus, HSV, adenovirus, adeno-associated virus, Semiliki Forest virus, cytomegalovirus, and vaccinia virus. The amount of nucleic acid encoding CD81 to be used is an amount that will express CD81 protein in an amount effective to inhibit proliferation of astrocytes, as defined above. These amounts may be readily determined by the skilled artisan.

[0073] The present invention is also directed to methods for inhibiting proliferation of astrocytic tumor cells, by contacting astrocytic tumor cells with an amount of CD81 effective to inhibit proliferation of astrocytic tumor cells. As used herein, the term "astrocytic tumor cells" refers to a tumorigenic form of astrocytes (*i.e.*, transformed astrocytes), and includes astrocytoma cells (*i.e.*, cells of all astrocytomas, including, without limitation, Grades I-IV astrocytomas, anaplastic astrocytoma, astroblastoma, astrocytoma fibrillare, astrocytoma protoplasmaticum, gemistocytic astrocytoma, and glioblastoma multiforme). As defined above, "CD81" includes a CD81 protein (p27), a CD81 analogue, and a CD81 derivative.

[0074] The methods of the present invention may be used to inhibit proliferation of astrocytic tumor cells *in vitro*, or *in vivo* in a subject. As used herein, the term "inhibit proliferation of astrocytic tumor cells" means inhibit cell division and growth of astrocytic tumor cells, and includes limiting the proliferative rate of astrocytic tumor cells. Inhibition of the growth and proliferation of astrocytic tumor cells may be detected by known procedures, including any of the methods, molecular procedures, and assays disclosed herein.

[0075] In accordance with the methods of the present invention, CD81 may be contacted with astrocytic tumor cells *in vitro*, or *in vivo* in a subject, by introducing the CD81 protein into the membranes of astrocytic tumor cells, or by introducing into the astrocytic tumor cells a nucleic acid encoding CD81 in a manner permitting expression of CD81 protein. The subject may be any animal, but is preferably a mammal (*e.g.*, humans, domestic animals, and commercial animals). More preferably, the subject is a human. The astrocytic tumor cells may be found in neural tissue and other tissue of the nervous system of the subject, either alone or with other types of cells, including, without limitation, neurons and oligodendroglia. Astrocytic tumor cells may be detected in tissue of the subject by standard detection methods readily determined from the known art, including, without limitation, immunological techniques (*e.g.*, immunohistochemical staining), fluorescence imaging techniques, and microscopic techniques.

[0076] CD81 protein may be introduced into the membranes of astrocytic tumor cells, either *in vitro* or *in vivo* in a subject, by known techniques used for the introduction of proteins (*e.g.*, liposome delivery), as described above. For liposome delivery, liposomal vesicles and liposome compositions may be prepared using a variety of conventional techniques, including those described above. CD81 protein may be incorporated into the layers of a liposome such that its extracellular domain extends outside the liposome, and its intracellular domain extends into the interior of the liposome. The liposome containing CD81 then may be fused with astrocytic tumor cells, in accordance with known methods of fusion of liposomes to cell membranes, such that the CD81 protein is delivered into the membrane of the astrocytic tumor cells. The amount of CD81 protein to be used is an amount effective to inhibit proliferation of astrocytic tumor cells, as defined above, and may be readily determined by the skilled artisan.

[0077] In the method of the present invention, CD81 also may be contacted with astrocytic tumor cells, either *in vitro* or *in vivo* in a subject, by introducing into a sufficient number of astrocytic tumor cells of the subject a nucleic acid encoding CD81, in a manner permitting expression of CD81. The nucleic acid may be introduced using conventional

procedures known in the art, including *in vivo* gene therapy, *ex vivo* gene therapy, and all other above-described procedures. Recombinant viral vectors suitable for gene therapy include all of the vectors described above. The amount of nucleic acid encoding CD81 to be used is an amount that will express CD81 protein in an amount effective to inhibit proliferation of astrocytic tumor cells, as defined above. These amounts may be readily determined by the skilled artisan.

[0078] The ability of CD81 to modulate astrocyte proliferation, and the absence of CD81 from astrocytic tumor cell lines, together suggest that CD81 may be useful for treating astrocytomas and other conditions associated with proliferation of astrocytic tumor cells. Furthermore, it is believed that CD81 would be effective either alone or in combination with therapeutic agents, such as chemotherapeutic agents or antiviral agents, which are typically used in the treatment of these conditions.

[0079] Accordingly, the present invention provides a method for treating a condition associated with proliferation of astrocytic tumor cells in a subject in need of treatment, comprising contacting astrocytic tumor cells in the subject with an amount of CD81 effective to inhibit proliferation of astrocytic tumor cells, thereby treating the condition. As described above, the subject may be any animal, but is preferably a mammal (*e.g.*, humans, domestic animals, and commercial animals). More preferably, the subject is a human.

[0080] As used herein, the term "conditions associated with proliferation of astrocytic tumor cells" includes pathologic proliferation of astrocytic tumor cells, such as astrocytoma cells, and other forms of neoplasia. The term "neoplasia", as further used herein, refers to the uncontrolled and progressive multiplication of astrocytic tumor cells under conditions that would not elicit, or would cause cessation of, multiplication of normal astrocytes. Neoplasia results in the formation of a "neoplasm", which is defined herein to mean any new and abnormal growth, particularly a new growth of tissue, in which the growth of cells is uncontrolled and progressive. Neoplasms include benign tumors and malignant tumors (*e.g.*, astrocytomas, such as Grades I-IV astrocytomas, anaplastic astrocytoma, astroblastoma, astrocytoma fibrillare, astrocytoma protoplasmaticum, gemistocytic astrocytoma, and glioblastoma multiforme, and other brain tumors). Malignant neoplasms are distinguished from benign in that the former show a greater degree of anaplasia, or loss of differentiation and orientation of cells, and have the properties of invasion and metastasis. Thus, neoplasia includes "cancer", which herein refers to a proliferation of astrocytic tumor cells having the unique trait of loss of normal controls, resulting in unregulated growth, lack of differentiation, local tissue invasion, and metastasis. In one embodiment of the

present invention, the condition associated with proliferation of astrocytic tumor cells is an astrocytoma.

[0081] In the treatment of a condition associated with proliferation of astrocytic tumor cells, CD81 may be contacted with astrocytic tumor cells by introducing the CD81 protein into the membranes of astrocytic tumor cells, in accordance with known methods (*e.g.*, liposome delivery), as described above. The amount of CD81 protein to be used is an amount effective to inhibit proliferation of astrocytic tumor cells, as defined above, and may be readily determined by the skilled artisan.

[0082] Alternatively, in accordance with known methods, including those described above, CD81 may be contacted with astrocytic tumor cells to treat a condition associated with a defect in astrocytic tumor cell proliferation by introducing into the astrocytic tumor cells a nucleic acid encoding CD81, in a manner permitting expression of CD81 protein. The nucleic acid may be introduced using conventional procedures known in the art, including *in vivo* gene therapy, *ex vivo* gene therapy, and all above-described procedures. Recombinant viral vectors suitable for gene therapy include all vectors described above. The amount of nucleic acid encoding CD81 to be used is an amount that will express CD81 protein in an amount effective to inhibit proliferation of astrocytic tumor cells, as defined above. These amounts may be readily determined by the skilled artisan.

[0083] The present invention further provides methods for inhibiting proliferation of astrocytes, comprising contacting astrocytes with a modulator of CD81 expression, in an amount effective to inhibit proliferation of astrocytes. The modulator may be a protein, polypeptide, peptide, nucleic acid (including DNA or RNA), antibody, Fab fragment, $F(ab')_2$ fragment, molecule, compound, antibiotic, drug, neuron, or other agent, as defined herein, that induces or upregulates CD81 expression. Examples of modulators include, without limitation, neurons, FK506, and other neuroimmunophilins.

[0084] Additional modulators of CD81 may be identified using a simple screening assay based on procedures described below. For example, to screen for candidate modulators of CD81, astrocytic tumor cells may be plated onto microtiter plates, then contacted with a library of drugs. Any resulting expression of CD81 may be detected using nucleic acid hybridization and/or immunological techniques known in the art, including an ELISA. Modulators of CD81 will be those drugs which induce or upregulate expression of CD81. In this manner, agents also may be screened for their ability to inhibit proliferation of astrocytes or astrocytic tumor cells using CD81

expression as an indicator that cell division or growth of astrocytes or astrocytic tumor cells is decreasing in rate, or has stopped.

[0085] The present invention further provides methods for inhibiting proliferation of astrocytic tumor cells, comprising contacting astrocytic tumor cells with a modulator of CD81 expression, in an amount effective to inhibit proliferation of astrocytic tumor cells. Examples of such modulators of CD81 expression include all of those described above. Additional modulators of CD81 may be screened in accordance with the above-described methods.

[0086] The present invention also provides methods for treating a condition associated with a defect in astrocyte proliferation in a subject in need of treatment. The methods comprise administering to the subject an amount of CD81 effective to treat the condition associated with a defect in cell proliferation. The subject may be any animal, but is preferably a mammal (*e.g.*, humans, domestic animals, and commercial animals). More preferably, the subject is a human.

[0087] As described above, examples of conditions associated with a defect in astrocyte proliferation include, without limitation, astrocytosis, glial scars, hyperplasia, neoplasia, and neuritic plaques (particularly those commonly found in Alzheimer's disease patients). Additionally, conditions associated with a defect in astrocyte proliferation may be caused by, or associated with, a variety of factors, including, without limitation, neuronal cell death and neural degeneration resulting from neurodegenerative diseases, CNS traumas, and the acquired secondary effects of non-neural dysfunction. Examples of neurodegenerative diseases, CNS traumas, and acquired secondary effects of non-neural dysfunction include all of those described above. In one embodiment of the present invention, the condition associated with a defect in astrocyte proliferation is astrocytosis.

[0088] The CD81 of the present invention is administered to a subject in need of treatment for a condition associated with a defect in astrocyte proliferation in an amount that is effective to treat the condition associated with a defect in astrocyte proliferation in the subject. As used herein, the phrase "effective to treat the condition associated with a defect in astrocyte proliferation" means effective to ameliorate or minimize the clinical impairment or symptoms of the condition associated with a defect in astrocyte proliferation. For example, where the condition associated with a defect in astrocyte proliferation is astrocytosis, the clinical impairment or symptoms of the astrocytosis may be ameliorated or minimized by reducing the mass of astrocytes produced by the astrocytosis, thereby minimizing any potential obstruction of axons which may occur. The amount of CD81 effective to treat a condition associated with a defect in astrocyte

proliferation in a subject in need of treatment therefor will vary depending upon the particular factors of each case, including the type of defect in astrocyte proliferation, the stage of the defect in astrocyte proliferation, the subject's weight, the severity of the subject's condition, and the method of administration. This amount can be readily determined by the skilled artisan.

[0089] According to the method of the present invention, CD81 may be administered to a human or animal subject by known procedures, including, without limitation, oral administration, parenteral administration, transdermal administration, and administration through an osmotic mini-pump. Preferably, the CD81 is administered parenterally, by intracranial, intraspinal, intrathecal, or subcutaneous injection. The CD81 of the present invention also may be administered to a subject in accordance with any of the above-described methods for effecting *in vivo* contact between astrocytes and CD81.

[0090] For oral administration, the formulation of CD81 may be presented as capsules, tablets, powders, granules, or as a suspension. The formulation may have conventional additives, such as lactose, mannitol, corn starch, or potato starch. The formulation also may be presented with binders, such as crystalline cellulose, cellulose derivatives, acacia, corn starch, or gelatins. Additionally, the formulation may be presented with disintegrators, such as corn starch, potato starch, or sodium carboxymethylcellulose. The formulation also may be presented with dibasic calcium phosphate anhydrous or sodium starch glycolate. Finally, the formulation may be presented with lubricants, such as talc or magnesium stearate.

[0091] For parenteral administration (*i.e.*, administration by injection through a route other than the alimentary canal), CD81 may be combined with a sterile aqueous solution that is preferably isotonic with the blood of the subject. Such a formulation may be prepared by dissolving a solid active ingredient in water containing physiologically-compatible substances, such as sodium chloride, glycine, and the like, and having a buffered pH compatible with physiological conditions, so as to produce an aqueous solution, then rendering said solution sterile. The formulations may be presented in unit or multi-dose containers, such as sealed ampoules or vials. The formulation may be delivered by any mode of injection, including, without limitation, epifascial, intracapsular, intracranial, intracutaneous, intrathecal, intramuscular, intraorbital, intraperitoneal, intraspinal, intrasternal, intravascular, intravenous, parenchymatous, or subcutaneous.

[0092] For transdermal administration, CD81 may be combined with skin penetration enhancers, such as propylene glycol, polyethylene glycol, isopropanol, ethanol, oleic acid, *N*-

methylpyrrolidone, and the like, which increase the permeability of the skin to the CD81, and permit the CD81 to penetrate through the skin and into the bloodstream. The CD81/enhancer compositions also may be further combined with a polymeric substance, such as ethylcellulose, hydroxypropyl cellulose, ethylene/vinylacetate, polyvinyl pyrrolidone, and the like, to provide the composition in gel form, which may be dissolved in solvent, such as methylene chloride, evaporated to the desired viscosity, and then applied to backing material to provide a patch. CD81 may be administered transdermally at the site in the subject where neural trauma has occurred, or where the defect in astrocyte proliferation is localized. Alternatively, CD81 may be administered transdermally at a site other than the affected area, in order to achieve systemic administration.

[0093] The CD81 of the present invention also may be released or delivered from an osmotic mini-pump or other time-release device. The release rate from an elementary osmotic mini-pump may be modulated with a microporous, fast-response gel disposed in the release orifice. An osmotic mini-pump would be useful for controlling release, or targeting delivery, of CD81.

[0094] The present invention also provides methods for treating a condition associated with proliferation of astrocytic tumor cells in a subject in need of treatment therefor. The methods comprise administering to the subject an amount of CD81 effective to treat the condition associated with proliferation of astrocytic tumor cells. The subject may be any animal, but is preferably a mammal (*e.g.*, humans, domestic animals, and commercial animals). More preferably, the subject is a human. As described above, examples of conditions associated with proliferation of astrocytic tumor cells include, without limitation, astrocytomas, brain tumors, and other forms of neoplasia. In one embodiment of the present invention, the condition associated with proliferation of astrocytic tumor cells is an astrocytoma.

[0095] The CD81 of the present invention is administered to a subject in need of treatment for a condition associated with proliferation of astrocytic tumor cells in an amount that is effective to treat the condition associated with proliferation of astrocytic tumor cells in the subject. As used herein, the phrase "effective to treat the condition associated with proliferation of astrocytic tumor cells" means effective to ameliorate or minimize the clinical impairment or symptoms of the condition associated with proliferation of astrocytic tumor cells.

[0096] For example, where the condition associated with proliferation of astrocytic tumor cells is an astrocytoma, the clinical impairment or symptoms of the astrocytoma may be

ameliorated or minimized by diminishing any pain or discomfort suffered by the subject; by extending the survival of the subject beyond that which would otherwise be expected in the absence of such treatment; by inhibiting or preventing the development or spread of the neoplasm; or by limiting, suspending, terminating, or otherwise controlling the maturation and proliferation of astrocytic tumor cells in the astrocytoma. The amount of CD81 effective to treat a condition associated with proliferation of astrocytic tumor cells in a subject in need of treatment will vary depending upon the particular factors of each case, including the type of condition associated with proliferation of astrocytic tumor cells, the stage of the condition associated with proliferation of astrocytic tumor cells, the subject's weight, the severity of the subject's condition, and the method of administration. This amount can be readily determined by the skilled artisan.

[0097] According to the method of the present invention, CD81 may be administered to a human or animal subject by known procedures, including, without limitation, oral administration, parenteral administration, transdermal administration, and administration through an osmotic mini-pump. Preferably, the CD81 is administered parenterally, by intracranial, intraspinal, intrathecal, or subcutaneous injection. The CD81 of the present invention also may be administered to a subject in accordance with any of the above-described methods for effecting *in vivo* contact between astrocytic tumor cells and CD81.

[0098] For oral administration, the formulation of CD81 may be presented as capsules, tablets, powders, granules, as a suspension, or in any of the above-described formulations. For parenteral administration, CD81 may be combined with a sterile aqueous solution which is preferably isotonic with the blood of the subject. Such a formulation may be prepared in accordance with the above-described method of preparation. The formulations for parenteral administration may be presented in unit or multi-dose containers, such as sealed ampoules or vials, and may be delivered by any of the modes of injection described above.

[0099] For transdermal administration, CD81 may be combined with skin penetration enhancers, such as those described above. The CD81/enhancer compositions also may be further combined with a polymeric substance, such as any of those described above, to provide the composition in gel form. CD81 may be administered transdermally at the site in the subject where astrocytic tumor cell proliferation has occurred. Alternatively, CD81 may be administered transdermally at a site other than the affected area, in order to achieve systemic administration. Finally, the CD81 of the present invention also may be released or delivered from an osmotic mini-pump or other time-release device, as described above.

[0100] The present invention further provides methods for treating a condition associated with a defect in astrocyte proliferation in a subject in need of treatment. The methods comprise administering to the subject a modulator of CD81 expression in an amount effective to induce or enhance expression of CD81 and treat a condition associated with a defect in astrocyte proliferation, as defined above, in the subject. Examples of such modulators of CD81 expression include all of those described above. Additional modulators of CD81 may be screened in accordance with the above-described methods. The modulator of CD81 may be administered to a subject in any of the formulations, and by any of the modes of administration, described above.

[0101] The present invention also provides methods for treating a condition associated with proliferation of astrocytic tumor cells in a subject in need of treatment. The methods comprise administering to the subject a modulator of CD81 expression in an amount effective to induce or enhance expression of CD81 and treat the condition associated with proliferation of astrocytic tumor cells, as defined above, in the subject. Examples of such modulators of CD81 expression include all of those described above. Additional modulators of CD81 may be screened in accordance with the above-described methods. The modulator of CD81 may be administered to a subject in any of the formulations, and by any of the modes of administration, described herein. Moreover, the modulator of CD81 also may be administered along with a chemotherapeutic agent, such as a ricin-conjugated CD81-binding protein.

[0102] In view of the foregoing, it is predicted that administration of CD81 will provide an effective treatment option for conditions associated with either a defect in astrocyte proliferation or a proliferation of astrocytic tumor cells. The therapies described herein offer real treatment options for inhibiting astrocyte and astrocytoma proliferation, without the massive side-effects and bystander effects that typically accompany the current treatment regimes. The population at risk for these conditions is large, and the needs currently are not being met.

[0103] The present invention further provides pharmaceutical compositions comprising CD81 and a pharmaceutically-acceptable carrier, wherein CD81 is present in an amount sufficient or effective to treat a condition associated with a defect in astrocyte proliferation, as defined above, in a subject to whom said pharmaceutical composition is administered. Such a pharmaceutical composition would be useful for administering CD81 to a subject in need of treatment for a condition associated with a defect in astrocyte proliferation, in order to treat said condition. The CD81 is provided to the subject in an amount that is effective to treat the condition associated with a defect in astrocyte proliferation, as defined above, in the subject. This

amount may be readily determined by the skilled artisan. The pharmaceutical composition may be administered to a subject in accordance with any of the methods of administration described above.

[0104] Formulations of the pharmaceutical composition of the present invention may be conveniently presented in unit dosage, and may be presented in oral dosage form (*e.g.*, CD81 and a pharmaceutically-acceptable carrier may be combined in an ampule, capsule, pill, powder, or tablet) or in a form suitable for injection. The pharmaceutically-acceptable carrier may be a solid, liquid, or gel. Furthermore, the pharmaceutically-acceptable carrier of the present invention must be "acceptable" in the sense of being compatible with the other ingredients of the composition, and not deleterious to the recipient thereof. Examples of acceptable pharmaceutical carriers include carboxymethylcellulose, crystalline cellulose, glycerin, gum arabic, lactose, magnesium stearate, methyl cellulose, polypeptides, powders, saline, sodium alginate, starch, sucrose, talc, and water, among others. The carrier selected will depend upon the route of administration, and the form in which CD81 is introduced.

[0105] The formulations of the present invention may be prepared by methods well known in the pharmaceutical art. For example, CD81 may be brought into association with a carrier or diluent, as an emulsion, suspension, or solution. Moreover, CD81 may be blended, at need, with another component, to the extent that such blending does not impair the object of the present invention. Such other component may be suitably selected in accordance with the purpose of use and type of formulation. Optionally, one or more accessory ingredients (*e.g.*, buffers, colorants, flavoring agents, surface active agents, and the like) also may be added.

[0106] The present invention is also directed to pharmaceutical compositions comprising a nucleic acid encoding CD81 and a pharmaceutically-acceptable carrier, wherein the nucleic acid expresses CD81 in an amount sufficient or effective to treat a condition associated with a defect in astrocyte proliferation, as defined above, in a subject to whom said pharmaceutical composition is administered. Such pharmaceutical compositions are useful for administering CD81 to a subject in need of treatment for a condition associated with a defect in astrocyte proliferation, in order to treat said condition in the subject. The nucleic acid is provided to the subject in an amount such that it expresses CD81 protein in an amount that is effective to treat a condition associated with a defect in astrocyte proliferation, as defined above, in the subject. These amounts may be readily determined by the skilled artisan. Additionally, the pharmaceutical composition may be

administered to a subject in accordance with any of the above-described methods of administration and introduction of nucleic acids.

[0107] Formulations of the pharmaceutical composition of the present invention may be conveniently presented in unit dosage, and may be presented in a form suitable for administration of nucleic acid (*e.g.*, by injection). The nucleic acid encoding CD81 may be presented in any form well known in the art for introduction of nucleic acids, including, without limitation, naked DNA, plasmid DNA, and vector DNA (including viral vectors, as described above), and may be prepared in accordance with methods well known in the arts of gene therapy and molecular genetics. In addition, the pharmaceutically-acceptable carrier of the present invention must be "acceptable" in the sense of being compatible with the other ingredients of the composition, and not deleterious to the recipient thereof. Examples of acceptable pharmaceutical carriers include carboxymethylcellulose, crystalline cellulose, glycerin, gum arabic, lactose, magnesium stearate, methyl cellulose, polypeptides, powders, saline, sodium alginate, starch, sucrose, talc, and water, among others. The carrier selected will depend upon the route of administration, and the form in which nucleic acid encoding CD81 is introduced.

[0108] The formulations of the pharmaceutical composition of the present invention may be prepared by methods well known in the pharmaceutical art. For example, nucleic acid encoding CD81 may be brought into association with a carrier or diluent, as an emulsion, suspension, or solution. Moreover, nucleic acid encoding CD81 may be blended, at need, with another component, to the extent that such blending does not impair the object of the present invention. Such other component may be suitably selected in accordance with the purpose of use and type of formulation. Optionally, one or more accessory ingredients (*e.g.*, buffers, colorants, surface active agents, and the like) also may be added.

[0109] The present invention further provides pharmaceutical compositions comprising CD81 and a pharmaceutically-acceptable carrier, wherein CD81 is present in an amount sufficient or effective to treat a condition associated with proliferation of astrocytic tumor cells, as defined above, in a subject to whom said pharmaceutical composition is administered. Such a pharmaceutical composition would be useful for administering CD81 to a subject in need of treatment for a condition associated with proliferation of astrocytic tumor cells, in order to treat said condition in the subject. The CD81 is provided to the subject in an amount that is effective to treat the condition associated with proliferation of astrocytic tumor cells, as defined above, in the subject. This amount may be readily determined by the skilled artisan. The pharmaceutical

composition may be administered to a subject in accordance with any of the methods of administration, and in any of the formulations, described above. The formulations of the present invention may be prepared in accordance with methods well known in the pharmaceutical art, including those described above.

[0110] The present invention is also directed to pharmaceutical compositions comprising a nucleic acid encoding CD81 and a pharmaceutically-acceptable carrier, wherein the nucleic acid expresses CD81 in an amount sufficient or effective to treat a condition associated with proliferation of astrocytic tumor cells, as defined above, in a subject to whom said pharmaceutical composition is administered. Such a pharmaceutical composition would be useful for administering CD81 to a subject in need of treatment for a condition associated with proliferation of astrocytic tumor cells, in order to treat said condition in the subject. The nucleic acid is provided to the subject in an amount such that it expresses CD81 protein in an amount that is effective to treat a condition associated with proliferation of astrocytic tumor cell, as defined above, in the subject. These amounts may be readily determined by the skilled artisan. Additionally, the pharmaceutical composition may be administered to a subject in accordance with any of the above-described methods of administration and introduction of nucleic acids, and in any of the formulations described above. The formulations of the pharmaceutical composition of the present invention may be prepared in accordance with methods well known in the pharmaceutical art, including those described above.

[0111] The present invention further provides methods for determining whether a subject has an astrocytoma. The methods comprise assaying for CD81 expression in a diagnostic sample of cells of astrocytic lineage of the subject, wherein no detection of expression of CD81 in cells of astrocytic lineage of the subject is diagnostic of an astrocytoma. The subject may be any animal, but is preferably a mammal (*e.g.*, humans, domestic animals, and commercial animals). More preferably, the subject is a human. As used herein, "CD81" includes CD81 protein, cDNA, and mRNA.

[0112] As used herein, "no detection of expression of CD81" means that CD81 is not present in astrocytic tumor cells of the subject at a detectable level. As further used herein, the term "cells of astrocytic lineage" includes astrocytes and astrocytic tumor cells, as defined above. It is also within the confines of the present invention to provide a method for confirming a diagnosis of astrocytoma in a subject, comprising assaying for CD81 expression a diagnostic

sample of cells of astrocytic lineage of the subject, wherein no detection of expression of CD81 in cells of astrocytic lineage of the subject is diagnostic of an astrocytoma.

[0113] According to the method of the present invention, the diagnostic sample of cells of astrocytic lineage of the subject may be assayed for CD81 expression *in vitro*, or *in vivo* in a subject. In accordance with the present invention, where the assay is performed *in vitro*, a diagnostic sample of cells of astrocytic lineage, or tissue containing cells of astrocytic lineage, may be removed from the subject using standard procedures, including biopsy and aspiration. Preferably, the diagnostic sample of cells or tissue is removed using multidirectional fine-needle aspiration biopsy (FNAB). This method of removal is preferred, as it is less invasive than a standard biopsy. The diagnostic sample taken from the subject may be, for example, any tissue known to have an astrocytoma, any tissue suspected of having an astrocytoma, or any tissue believed not to have an astrocytoma.

[0114] Protein may be isolated and purified from the diagnostic sample of the present invention using standard methods known in the art, including, without limitation, extraction from a tissue (*e.g.*, with a detergent that solubilizes the protein) where necessary, followed by affinity purification on a column, chromatography (*e.g.*, FTLC and HPLC), immunoprecipitation (with an antibody to CD81), and precipitation (*e.g.*, with isopropanol and a reagent such as Trizol). Isolation and purification of the protein may be followed by electrophoresis (*e.g.*, on an SDS-polyacrylamide gel). Nucleic acid may be isolated from a diagnostic sample using standard techniques known to one of skill in the art.

[0115] In accordance with the method of the present invention, an astrocytoma in a subject may be diagnosed by assaying a diagnostic sample of the subject for expression of CD81. Because CD81 is generally expressed in cells of astrocytic lineage from healthy, nondiseased subjects (*i.e.*, those who do not have an astrocytoma), no detection of CD81 expression in a diagnostic sample of cells of astrocytic lineage of a subject is diagnostic of an astrocytoma. As used herein, "expression" means the transcription of the CD81 gene into at least one mRNA transcript, or the translation of at least one mRNA into a CD81 protein, as defined above. Accordingly, a diagnostic sample may be assayed for CD81 expression by assaying for CD81 protein (as defined above), cDNA, or mRNA. The appropriate form of CD81 will be apparent based on the particular techniques discussed herein.

[0116] In these methods, a diagnostic sample of cells of astrocytic lineage a subject may be assayed for CD81 expression, and CD81 expression may be detected in a diagnostic sample,

using assays and detection methods readily determined from the known art, including, without limitation, immunological techniques, hybridization analysis, fluorescence imaging techniques, and/or radiation detection. For example, astrocytes or cells that are removed from the subject using FNAB may be analyzed using immunocytofluorometry (FACS analysis). In another embodiment of the present invention, the diagnostic sample is assayed for expression of CD81 using Northern blot analysis of CD81 mRNA extracted from cells of astrocytic lineage.

[0117] According to these methods, a diagnostic sample of the subject may be assayed for CD81 expression using an agent reactive with CD81. As used herein, "reactive" means the agent has affinity for, binds to, or is directed against CD81. As further used herein, an "agent" shall include a protein, polypeptide, peptide, nucleic acid (including DNA or RNA), antibody, Fab fragment, $F(ab')_2$ fragment, molecule, compound, antibiotic, drug, and any combinations thereof. Moreover, an agent reactive with CD81 may be either natural or synthetic. The agent may be in the form of an antibody, a Fab fragment, an $F(ab')_2$ fragment, a peptide, a polypeptide, a protein, and any combinations thereof. A Fab fragment is a univalent, antigen-binding fragment of an antibody, which is produced by papain digestion. An $F(ab')_2$ fragment is a divalent antigen-binding fragment of an antibody, which is produced by pepsin digestion. Preferably, the agent is a high-affinity antibody labeled with a detectable marker. Where the agent is an antibody, the absence of expression of CD81 may be detected from binding studies using one or more antibodies immunoreactive with CD81, along with standard immunological detection techniques, such as Western blotting.

[0118] As used herein, the antibody of the present invention may be polyclonal or monoclonal, and may be produced by techniques well known to those skilled in the art. Polyclonal antibody, for example, may be produced by immunizing a mouse, rabbit, or rat with purified CD81. Monoclonal antibody then may be produced by removing the spleen from the immunized mouse, and fusing the spleen cells with myeloma cells to form a hybridoma which, when grown in culture, will produce a monoclonal antibody. Monoclonal antibodies that are reactive with CD81 also may be obtained from Pharmingen (San Diego, CA) (*e.g.*, mAb 2F7) and Boehringer (Mannheim, Germany) (*e.g.*, mAbs Eat1 and Eat2).

[0119] The antibodies used herein may be labeled with a detectable marker. Labeling of the antibody may be accomplished using one of the variety of different chemiluminescent and radioactive labels known in the art. The detectable marker of the present invention may be, for example, a nonradioactive or fluorescent marker, such as biotin, fluorescein (FITC), acridine,

cholesterol, or carboxy-X-rhodamine, which can be detected using fluorescence and other imaging techniques readily known in the art. Alternatively, the detectable marker may be a radioactive marker, including, for example, a radioisotope. The radioisotope may be any isotope that emits detectable radiation, such as ^{35}S, ^{32}P, or ^{3}H. Radioactivity emitted by the radioisotope can be detected by techniques well known in the art. For example, gamma emission from the radioisotope may be detected using gamma imaging techniques, particularly scintigraphic imaging. Preferably, the agent of the present invention is a high-affinity antibody labeled with a detectable marker. The antibodies of the present invention also may be incorporated into kits that include an appropriate labeling system, buffers, and other necessary reagents for use in a variety of detection and diagnostic applications.

[0120] Where the agent of the present invention is an antibody reactive with CD81, a diagnostic sample taken from the subject may be purified by passage through an affinity column which contains CD81 antibody as a ligand attached to a solid support such as an insoluble organic polymer in the form of a bead, gel, or plate. The antibody attached to the solid support may be used in the form of a column. Examples of suitable solid supports include, without limitation, agarose, cellulose, dextran, polyacrylamide, polystyrene, sepharose, or other insoluble organic polymers. The CD81 antibody may be further attached to the solid support through a spacer molecule, if desired. Appropriate binding conditions (*e.g.*, temperature, pH, and salt concentration) may be readily determined by the skilled artisan. In a preferred embodiment, the CD81 antibody is attached to a sepharose column, such as Sepharose 4B.

[0121] Where the agent is an antibody, a diagnostic sample of the subject may be assayed for CD81 expression using binding studies that utilize one or more antibodies immunoreactive with CD81, along with standard immunological detection techniques. For example, the CD81 protein eluted from the affinity column may be subjected to an ELISA assay, Western blot analysis, flow cytometry, or any other immunostaining method employing an antigen-antibody interaction. Preferably, the diagnostic sample is assayed for CD81 expression using Western blotting.

[0122] Alternatively, a diagnostic sample of cells of astrocytic lineage of a subject may be assayed for CD81 expression using hybridization analysis of nucleic acid extracted from a sample of cells of astrocytic lineage, or tissue containing cells of astrocytic lineage, taken from the subject. According to this method of the present invention, the hybridization analysis may be conducted using Northern blot analysis of mRNA. This method also may be conducted by

performing a Southern blot analysis of DNA using one or more nucleic acid probes which hybridize to nucleic acid encoding CD81. The nucleic acid probes may be prepared by a variety of techniques known to those skilled in the art, including, without limitation, the following: restriction enzyme digestion of CD81 nucleic acid; and automated synthesis of oligonucleotides having sequences that correspond to selected portions of the nucleotide sequence of the CD81 nucleic acid, using commercially-available oligonucleotide synthesizers, such as the Applied Biosystems Model 392 DNA/RNA synthesizer.

[0123] The nucleic acid probes used in the present invention may be DNA or RNA, and may vary in length from about 8 nucleotides to the entire length of the CD81 nucleic acid. The CD81 nucleic acid used in the probes may be derived from mammalian CD81. The nucleotide sequences for both rat, mouse, and human CD81 are known (19). Using these sequences as probes, the skilled artisan could readily clone corresponding CD81 cDNA from other species. In addition, the nucleic acid probes of the present invention may be labeled with one or more detectable markers. Labeling of the nucleic acid probes may be accomplished using one of a number of methods known in the art (*e.g.*, nick translation, end labeling, fill-in end labeling, polynucleotide kinase exchange reaction, random priming, or SP6 polymerase for riboprobe preparation), along with one of a variety of labels (*e.g.*, radioactive labels, such as ^{35}S, ^{32}P, or ^{3}H, or nonradioactive labels, such as biotin, fluorescein (FITC), acridine, cholesterol, or carboxy-X-rhodamine (ROX)). Combinations of two or more nucleic acid probes (or primers), corresponding to different or overlapping regions of the CD81 nucleic acid, also may be used to detect expression of CD81, using, for example, PCR or RT-PCR, and may be included in kits for use in a variety of detection and diagnostic applications.

[0124] It is contemplated that the diagnostic sample in the present invention frequently will be assayed for CD81 expression not by the subject, nor by his/her consulting physician, but by a laboratory technician or other clinician. Accordingly, the method of the present invention further comprises providing to a subject's consulting physician a report of the results obtained upon assaying a diagnostic sample of the subject for CD81 expression.

[0125] The present invention also provides methods for treating astrocytoma in a subject or patient. The subject may be any animal, but is preferably a mammal (*e.g.*, humans, domestic animals, and commercial animals). More preferably, the subject is a human. The methods comprise the steps of: (a) diagnosing an astrocytoma in the subject or patient by detecting an absence of expression of CD81 in cells of astrocytic lineage of the subject or patient; and (b)

treating the astrocytoma diagnosed in the subject or patient. The absence of expression of CD81 in cells of astrocytic lineage of the subject or patient may be detected by any of the methods described above. The astrocytoma diagnosed in the subject or patient may be treated by any method or combination of methods commonly used to treat astrocytoma, including, without limitation, surgery, radiotherapy, chemotherapy, immunotherapy, and systemic therapy. Preferably, however, an astrocytoma which is diagnosed in accordance with the method described herein is treated by administering CD81 to the subject or patient, as described above.

[0126] It is also within the confines of the present invention to use detected levels of CD81 expression as a clinical or pathologic staging tool, to determine which treatment options may be appropriate. In particular, detection of CD81 expression may be used to determine whether any of the treatment methods of the present invention is appropriate. Moreover, detected levels of CD81 expression may be used to grade brain tumors, particularly astrocytomas.

[0127] The present invention further provides methods for assessing the efficacy of astrocytoma therapy in a subject who has undergone or is undergoing treatment for astrocytoma. The subject may be any animal, but is preferably a mammal (*e.g.*, humans, domestic animals, and commercial animals). More preferably, the subject is a human. The method of the present invention comprises assaying for CD81 expression a diagnostic sample of astrocytic tumor cells of the subject, wherein no detection of expression of CD81 in astrocytic tumor cells of the subject is indicative of unsuccessful astrocytoma therapy. The diagnostic sample may be any of those described above, and may be assayed for expression of CD81 either *in vitro* or *in vivo* in a subject. In addition, the diagnostic sample may be assayed for expression of CD81 using all of the various assays and methods of detection described above. This method of the present invention provides a means of monitoring the effectiveness of astrocytoma therapy by permitting the periodic assessment of levels of CD81 expression in astrocytic tumor cells of the subject.

[0128] According to these methods, a diagnostic sample of astrocytic tumor cells of a subject may be assayed, and levels of CD81 expression may be assessed, at any time following the initiation of therapy to treat an astrocytoma. For example, levels of CD81 expression may be assessed while the subject or patient is still undergoing treatment for the astrocytoma. Where expression of CD81 remains absent from astrocytic tumor cells of the subject, a physician may choose to continue with the astrocytoma treatment. Where levels of CD81 expression become detectable in astrocytic tumor cells of the subject, and then increase through successive assessments, it may be an indication that the astrocytoma treatment is working, and that treatment

doses could be decreased or even ceased. Where levels of CD81 do not noticeably increase through successive assessments, it may be an indication that the astrocytoma treatment is not working, and that treatment doses could be increased. Where CD81 expression is eventually detected in astrocytic tumor cells of a subject or patient at a level expected for normal, non-diseased astrocytes, a physician may conclude that the astrocytoma treatment has been successful, and that such treatment may cease. It is also within the confines of the present invention to assess levels of CD81 expression following completion of the subject's or patient's astrocytoma treatment, in order to determine whether the astrocytoma has recurred in the subject or patient. Furthermore, it is within the confines of the present invention to use assessed levels of CD81 expression as a clinical or pathologic staging tool, to determine the extent of astrocytoma in the subject or patient, to determine appropriate treatment options, and to provide prognostic information.

[0129] The present invention also provides methods for enhancing survival of neurons, comprising contacting the neurons with CD81 protein or a CD81 derivative, in an amount effective to enhance survival of the neurons. As used herein, "neurons" are any of the conducting or nerve cells of the nervous system that typically consist of a cell body (perikaryon) that contains the nucleus and surrounding cytoplasm; several short, radiating processes (dendrites); and one long process (the axon), which terminates in twig-like branches (telodendrons), and which may have branches (collaterals) projecting along its course. In the method of the present invention, the neurons may be contained in neural tissue and other tissue of the nervous system, either alone or with other types of neural cells, including, without limitation, astrocytes and oligodendroglia. Neurons may be detected in tissue by standard detection methods readily determined from the known art, examples of which include, without limitation, immunological techniques (*e.g.*, immunohistochemical staining), fluorescence imaging techniques, and microscopic techniques.

[0130] As further used herein, the term "enhancing survival of neurons" means the full or partial protection of neurons from damage, death, degeneration, demyelination, or injury. Enhancement of the survival of neurons may be measured or detected by known procedures, including any of the methods, molecular procedures, and assays disclosed herein.

[0131] Additionally, unless otherwise indicated, "CD81", as used herein, includes both a CD81 protein (p27) and a CD81 analogue, as defined above. Additionally, as used herein, a "CD81 derivative" is a chemical substance derived from CD81, either directly or by modification, truncation, or partial substitution. For example, the CD81 derivative for use in the present

invention may be the extracellular domain (ECD) of CD81. In addition, the CD81 derivative of the present invention may be the mimetics, GM1109 or GM1416, as disclosed herein. Since they are smaller than CD81, the derivatives of CD81 more easily pass through the blood-brain barrier, thereby providing suitable therapeutics for enhancing survival of neurons *in vivo*.

[0132] These methods may be used to enhance survival of neurons *in vitro*, or *in vivo* in a subject. The CD81 or CD81 derivative may be contacted with neurons *in vitro*, or *in vivo* in a subject, by introducing to the neurons the CD81 or CD81-derivative protein, or by introducing to the neurons a nucleic acid encoding CD81 or the CD81 derivative in a manner permitting expression of the CD81 or CD81-derivative protein. As described above, the subject may be any animal, but is preferably a mammal (*e.g.*, humans, domestic animals, and commercial animals). More preferably, the subject is a human.

[0133] CD81 or a CD81 derivative may be contacted with neurons *in vitro* by adding CD81 protein or a CD81-derivative protein directly to the culture medium. Alternatively, CD81 or a CD81 derivative may be contacted with neurons *in vivo* in a subject by introducing into, or administering to, the subject the CD81 protein or a CD81-derivative protein. The CD81 protein or the CD81-derivative protein may be contacted with neurons, either *in vitro* or *in vivo* in a subject, by known techniques used for the introduction and administration of proteins and other drugs, including, for example, injection and transfusion. The amount of CD81 protein or CD81-derivative protein to be used is an amount effective to enhance survival of neurons, as defined above, and may be readily determined by the skilled artisan.

[0134] It may be desirable to enhance the survival of neurons *in vitro* when preparing nervous tissue for transplantation, diagnostics, drug screening, and the like. In respect of *in vivo* treatment, the ability of CD81 or a CD81 derivative to enhance the survival of neurons renders CD81 and its derivatives particularly useful for treating neural degeneration in a subject. As used herein, the term "neural degeneration" means a condition of deterioration of the neuron, wherein the neuron changes to a lower or less functionally-active form. It is believed that, by enhancing the survival of neurons, CD81 or a CD81 derivative will be useful for the treatment of neural degeneration. It is further believed that CD81 or a CD81 derivative would be effective either alone or in combination with therapeutic agents, such as chemotherapeutic agents or antiviral agents, which are typically used in the treatment of neural degeneration.

[0135] Accordingly, the present invention provides methods for treating neural degeneration in a subject in need of treatment, by contacting neurons in the subject with an

amount of CD81 or a CD81 derivative effective to enhance survival of neurons, thereby treating the neural degeneration. As described above, the subject may be any animal, but is preferably a mammal (*e.g.*, humans, domestic animals, and commercial animals). More preferably, the subject is a human.

[0136] Examples of neural degeneration which may be treated by the method of the present invention include, without limitation, primary neurologic conditions (*e.g.*, neurodegenerative diseases), as well as acquired secondary effects of non-neural dysfunction (*e.g.*, neural loss secondary to degenerative, pathologic, or traumatic events). Examples of primary neurologic conditions or neurodegenerative diseases include, without limitation, Alzheimer's disease, amyotrophic lateral sclerosis (Lou Gehrig's Disease), Binswanger's disease, Huntington's chorea, multiple sclerosis, myasthenia gravis, Parkinson's disease, and Pick's disease. Examples of acquired secondary effects of non-neural dysfunction include, without limitation, cerebral palsy, congenital hydrocephalus, muscular dystrophy, stroke, and vascular dementia, as well as neural degeneration resulting from any of the following: an injury associated with cerebral hemorrhage, developmental disorders (*e.g.*, a defect of the brain, such as congenital hydrocephalus, or a defect of the spinal cord, such as spina bifida), diabetic encephalopathy, hypertensive encephalopathy, intracranial aneurysms, ischemia, kidney dysfunction, subarachnoid hemorrhage, trauma to the brain and spinal cord, the treatment of therapeutic agents such as chemotherapy agents and antiviral agents, vascular lesions of the brain and spinal cord, and other diseases or conditions prone to result in neural degeneration.

[0137] It is also within the confines of the present invention that the method described herein may be used to treat neural degeneration that is associated with a demyelinating condition. Examples of demyelinating conditions include, without limitation, acute disseminated encephalomyelitis (ADEM), acute transverse myelitis, acute viral encephalitis, adrenoleukodystrophy (ALD), adrenomyeloneuropathy, AIDS-vacuolar myelopathy, HTLV-associated myelopathy, Leber's hereditary optic atrophy, multiple sclerosis (MS), progressive multifocal leukoencephalopathy (PML), subacute sclerosing panencephalitis, and tropical spastic paraparesis.

[0138] In the treatment of neural degeneration in a subject, CD81 or a CD81 derivative may be contacted with neurons *in vivo*, within a subject who has neural degeneration, by administering to the subject CD81 or a CD81 derivative, in accordance with known methods of protein administration. The amount of CD81 or CD81-derivative protein to be used is an amount

effective to enhance survival of neurons, as defined above, and may be readily determined by the skilled artisan.

[0139] To effect contacting *in vivo* in a subject, CD81-derivative proteins (*e.g.*, the ECD of CD81, or the mimetics, GM1109 and GM1416) may be more appropriate for administration to the subject, as these smaller proteins can more readily cross the blood-brain barrier. Moreover, when target neurons are localized to a particular portion of the body of a subject, it may be desirable to introduce the CD81 or CD81-derivative protein directly to the tissue by injection or by some other means (*e.g.*, by introducing CD81 or CD81-derivative protein into the blood or another body fluid).

[0140] The CD81 or CD81 derivative may be administered to the subject to treat neural degeneration by any of the modes of administration, and in any of the formulations, described above. For example, the CD81 or CD81 derivative may be administered to the subject by oral administration, parenteral administration, sublingual administration, transdermal administration, or by osmotic pump. In one embodiment of the present invention, the CD81 or CD81 derivative is administered to the subject by oral administration or transdermal administration.

[0141] As disclosed herein, the novel transmembrane tyrosine kinase NrS1, which is located at the neuronal surface, is rapidly phosphorylated in response to CD81 signaling. This rapid phosphorylation ultimately results in forward signaling to the astrocyte. Additionally, the binding of the CD81, or a mimetic thereof, to NrS1 on the neuronal surface drives cleavage of neuregulin (Nrg) and the translocation of the intracellular domain of neuregulin (ICD_{Nrg}) to the neuronal nucleus, thereby promoting neuronal survival. Thus, there is also reverse signaling to the neuron. The nucleotide sequence of a NrS1 gene (previously identified as EHK1) is provided in FIG. 27; a previously unidentified exon of NrS1 that is 5' to the published EHK1 sequence is provided in FIG. 29. The corresponding amino acid sequence is provided in FIG. 28. The skilled artisan would understand that the novel NrS1 exon would be useful, e.g., as a probe for NrS1.

[0142] Accordingly, the present invention is further directed to methods for treating neural degeneration in a subject in need of treatment therefor. The methods comprise activating NrS1 in the subject. The subject may be any animal, but is preferably a mammal (*e.g.*, humans, domestic animals, and commercial animals). More preferably, the subject is a human. NrS1 in a subject may be activated by targeting NrS1 directly. NrS1 in a subject also may be activated indirectly, by targeting an enzyme or other endogenous molecule that regulates or modulates the functions or levels of NrS1 in the subject.

[0143] Unless otherwise indicated, "NrS1" includes both an NrS1 protein as characterized herein (having the amino acid sequence provided in FIG. 28), and an "NrS1 analogue". An "NrS1 analogue" is a functional variant of the NrS1 protein, having NrS1-protein biological activity, that has 60% or greater (preferably, 70% or greater; more preferably 80% or greater, even more preferably 90% or greater; even more preferably 95% or greater; and most preferably 97% or greater) amino-acid-sequence homology with the NrS1 protein, as well as a fragment of the NrS1 protein having NrS1-protein biological activity. The skilled artisan could identify such analogs based on functional studies with similar tyrosine kinases. As further used herein, the term "NrS1-protein biological activity" refers to protein activity which modulates and enhances survival of neurons, as disclosed herein. NrS1 may be produced synthetically or recombinantly, or may be isolated from native cells; however, it is preferably produced synthetically, using conventional techniques and cDNA encoding NrS1.

[0144] As defined above, the term "neural degeneration" means a condition of deterioration of the neuron, wherein the neuron changes to a lower or less functionally-active form. Neural degenerations which may be treated by the method of the present invention include any of those described above. For example, the neural degeneration may be a primary neurologic condition (*e.g.*, a neurodegenerative disease), as well as an acquired secondary effect of non-neural dysfunction (*e.g.*, neural loss secondary to degenerative, pathologic, or traumatic events). Examples of primary neurologic conditions, neurodegenerative diseases, and acquired secondary effects of non-neural dysfunction include all of those described above. It is also within the confines of the present invention that the method described herein may be used to treat neural degeneration that is associated with a demyelinating condition, including all of the above-described demyelinating conditions.

[0145] Additionally, as used herein, the term "activating NrS1" means stimulating or inducing the functions of NrS1 in the subject, particularly the cleavage of Nrg and the translocation of the ICD_{Nrg} to the neuronal nucleus as a result of the binding of CD81, or a derivative or mimetic thereof, to the NrS1 receptor at the neuronal surface, thereby promoting neuronal survival. In the method of the present invention, NrS1 in a subject may be activated, for example, by administering to the subject CD81 protein or a CD81-derivative protein. Examples of suitable CD81 derivatives include the large ECD of CD81 and the mimetics, GM1109 and GM1416.

[0146] The CD81 or CD81 derivative of the present invention may be administered to a subject in need of treatment for neural degeneration in an amount which is effective to treat the neural degeneration in the subject. As used herein, the phrase "effective to treat the neural degeneration" means effective to ameliorate or minimize the clinical impairment or symptoms of the neural degeneration. For example, where the neural degeneration is Alzheimer's disease, a neurodegenerative disease, the clinical impairment or symptoms of the neural degeneration may be ameliorated or minimized by reducing the amount of neurons lost within the cerebral cortex, hippocampus, subcortical structures, locus caeruleus, and nucleus raphae dorsalis, thereby minimizing or attenuating the progressive loss of cognitive function and the development of senile plaques and neurofibrillary tangles.

[0147] The amount of CD81 or CD81 derivative effective to treat neural degeneration in a subject in need of treatment will vary depending upon the particular factors of each case, including the type of neural degeneration, the stage of the neural degeneration, the subject's weight, the severity of the subject's condition, and the method of administration. This amount can be readily determined by the skilled artisan.

[0148] According to these methods, the CD81 protein or CD81-protein derivative may be administered to a human or animal subject by known procedures, including all of the modes of administration described above, and in all of the formulations described above. In a preferred embodiment of the present invention, the CD81 or CD81 derivative is administered by oral or transdermal administration. The CD81 and CD81 derivative of the present invention also may be administered to a subject in accordance with any of the above-described methods for effecting *in vivo* contact between neurons and CD81.

[0149] As disclosed herein, the inventor has discovered that full astrocyte differentiation depends upon CD81-NrS1 signaling. In view of this discovery, it is expected that NrS1 will provide a means for regulating astrocyte growth and proliferation. Accordingly, the present invention further provides methods for inhibiting proliferation of astrocytes. The methods comprise contacting the astrocytes with an amount of NrS1 effective to inhibit proliferation of the astrocytes. Unless otherwise indicated, "NrS1" includes both an NrS1 protein and an NrS1 analogue, as defined above.

[0150] These methods may be used to inhibit proliferation of astrocytes *in vitro*, or *in vivo* in a subject. As used herein, the term "inhibit proliferation of astrocytes", as defined above, means inhibit cell division and growth of astrocytes, and includes limiting the proliferative rate of

astrocytes, as disclosed herein. Inhibition of the growth and proliferation of astrocytes may be detected by known procedures, including any of the methods, molecular procedures, and assays disclosed herein.

[0151] In accordance these methods, NrS1 may be contacted with astrocytes *in vitro*, or *in vivo* in a subject, by introducing to the astrocytes the NrS1 protein itself, or by introducing to the astrocytes a nucleic acid encoding NrS1 in a manner permitting expression of NrS1 protein. The subject may be any animal, but is preferably a mammal (*e.g.*, humans, domestic animals, and commercial animals). More preferably, the subject is a human. The astrocytes may be contained in neural tissue and other tissue of the nervous system, either alone or with other types of neural cells, including, without limitation, neurons and oligodendroglia. Astrocytes may be detected in tissue by standard detection methods readily determined from the known art, including, without limitation, immunological techniques (*e.g.*, immunohistochemical staining), fluorescence imaging techniques, and microscopic techniques.

[0152] NrS1 may be contacted with astrocytes *in vitro* by adding NrS1 protein directly to the culture medium. Alternatively, NrS1 may be contacted with astrocytes *in vivo* in a subject by introducing into, or administering to, the subject the NrS1 protein. The NrS1 protein of the present invention may be contacted with astrocytes, either *in vitro* or *in vivo* in a subject, by known techniques used for the introduction and administration of proteins and other drugs, including, for example, injection and transfusion. When target astrocytes are localized to a particular portion of the body of the subject, it may be desirable to introduce the NrS1 protein directly to the tissue by injection or by some other means (*e.g.*, by introducing NrS1 protein into the blood or another body fluid). The amount of NrS1 protein to be used is an amount effective to inhibit proliferation of the astrocytes, as defined above, and may be readily determined by the skilled artisan.

[0153] It may be desirable to inhibit the proliferation of astrocytes *in vitro* when preparing nervous tissue for transplantation, diagnostics, drug screening, and the like. In respect of *in vivo* treatment, the ability of NrS1 to inhibit the proliferation of astrocytes renders NrS1 particularly useful for treating conditions associated with defects in astrocyte proliferation in a subject. As defined above, the term "defect in astrocyte proliferation" includes pathologic proliferation of astrocytes in a particular tissue, as compared with normal proliferation in the same type of tissue. It is believed that, by inhibiting the proliferation of astrocytes, NrS1 will be useful for the treatment of conditions associated with defects in astrocyte proliferation. It is further believed

that NrS1 would be effective either alone or in combination with therapeutic agents, such as chemotherapeutic agents or antiviral agents, which are typically used in the treatment of conditions associated with defects in astrocyte proliferation.

[0154] Accordingly, the present invention provides methods for treating a condition associated with a defect in astrocyte proliferation in a subject in need of treatment. The methods comprise contacting astrocytes in the subject with an amount of NrS1 effective to inhibit proliferation of astrocytes, thereby treating the condition associated with a defect in astrocyte proliferation. As described above, the subject may be any animal, but is preferably a mammal (*e.g.*, humans, domestic animals, and commercial animals). More preferably, the subject is a human.

[0155] In the method of the present invention, the condition associated with a defect in astrocyte proliferation may be any of those described above, including, without limitation, astrocytosis, glial scars, hyperplasia, neoplasia, and neuritic plaques (particularly those commonly found in Alzheimer's disease patients). Such conditions associated with a defect in astrocyte proliferation may be caused by a variety of factors, including any of those described above. In one embodiment of the present invention, the defect in astrocyte proliferation is astrocytosis. In another embodiment of the present invention, the defect in astrocyte proliferation is a neuritic plaque.

[0156] In the treatment of a condition associated with a defect in astrocyte proliferation in a subject, NrS1 may be contacted with astrocytes *in vivo*, within a subject who has a condition associated with a defect in astrocyte proliferation, by administering NrS1 to the subject, in accordance with known methods of protein administration. The amount of NrS1protein to be used is an amount effective to inhibit proliferation of astrocytes, as defined above, and may be readily determined by the skilled artisan.

[0157] The NrS1 may be administered to the subject by any of the modes of administration, and in any of the formulations, described above. For example, the NrS1 may be administered to the subject by oral administration, parenteral administration, sublingual administration, transdermal administration, or osmotic pump. In one embodiment of the present invention, the NrS1 is administered to the subject by oral administration or transdermal administration.

[0158] The present invention is also directed to methods for treating a condition associated with a defect in astrocyte proliferation in a subject in need of treatment. These

methods comprise activating CD81 in the subject. Unless otherwise indicated, CD81 includes both a CD81 protein and a CD81 analogue, as defined above. As used herein, the term "activating CD81" means stimulating or inducing the functions of CD81 in the subject, particularly the modulation or regulation of astrocyte growth and the induction of astrocyte growth arrest. CD81 in a subject may be activated by targeting CD81 directly. CD81 in a subject also may be activated indirectly, by targeting an enzyme or other endogenous molecule that regulates or modulates the functions or levels of CD81 in the subject. The subject may be any animal, but is preferably a mammal (*e.g.*, humans, domestic animals, and commercial animals). More preferably, the subject is a human.

[0159] In the method of the present invention, CD81 in a subject may be activated, for example, by administering NrS1 protein or NrS1 derivative to the subject. The NrS1 protein or NrS1 derivative of the present invention is administered to a subject in need of treatment for a condition associated with a defect in astrocyte proliferation in an amount which is effective to treat the condition associated with a defect in astrocyte proliferation in the subject. The condition associated with a defect in astrocyte proliferation may be any of those described above, include, without limitation, astrocytosis, glial scars, hyperplasia, neoplasia, and neuritic plaques (particularly those commonly found in Alzheimer's disease patients). Additionally, the condition associated with a defect in astrocyte proliferation may be caused by a variety of factors, including any of those described above. In one embodiment of the present invention, the defect in astrocyte proliferation is astrocytosis. In another embodiment of the present invention, the defect in astrocyte proliferation is a neuritic plaque.

[0160] As used herein, the phrase "effective to treat the condition associated with a defect in astrocyte proliferation" means effective to ameliorate or minimize the clinical impairment or symptoms of the condition associated with a defect in astrocyte proliferation. For example, where the condition associated with a defect in astrocyte proliferation is astrocytosis, the clinical impairment or symptoms of the condition may be ameliorated or minimized by reducing the mass of astrocytes produced by the astrocytosis, thereby minimizing any potential obstruction of axons which may occur.

[0161] The amount of NrS1 protein or NrS1 derivative effective to treat a condition associated with a defect in astrocyte proliferation in a subject in need of treatment will vary depending upon the particular factors of each case, including the type of defect in astrocyte proliferation, the stage of the defect in astrocyte proliferation, the subject's weight, the severity of

the subject's condition, and the method of administration. This amount can be readily determined by the skilled artisan.

[0162] According to the method of the present invention, the NrS1 protein or NrS1 derivative may be administered to a human or animal subject by known procedures, including all of the modes of administration described above, and in all formulations described above. In a preferred embodiment of the present invention, the NrS1 protein or NrS1 derivative is administered by oral or transdermal administration. The NrS1 protein or NrS1 derivative of the present invention also may be administered to a subject in accordance with any of the above-described methods for effecting *in vivo* contact between astrocytes and a protein.

[0163] The present invention is also directed to pharmaceutical compositions comprising NrS1 and a pharmaceutically-acceptable carrier, wherein NrS1 is present in an amount sufficient or effective to treat neural degeneration, as defined above, in a subject to whom said pharmaceutical composition is administered. Such pharmaceutical compositions are useful for administering NrS1 to a subject in need of treatment for neural degeneration, in order to treat the neural degeneration in the subject. The NrS1 is provided to the subject in an amount that is effective to treat the neural degeneration, as defined above, in the subject. This amount may be readily determined by the skilled artisan. The pharmaceutical compositions may be administered to a subject in accordance with any of the methods of administration, and in any of the formulations, described above.

[0164] Formulations of the pharmaceutical composition of the present invention may be conveniently presented in unit dosage, and may be presented in oral dosage form (*e.g.*, NrS1 and a pharmaceutically-acceptable carrier may be combined in an ampule, capsule, pill, powder, or tablet) or in a form suitable for injection. The pharmaceutically-acceptable carrier may be a solid, liquid, or gel. Furthermore, the pharmaceutically-acceptable carrier of the present invention must be "acceptable" in the sense of being compatible with the other ingredients of the composition, and not deleterious to the recipient thereof. Examples of acceptable pharmaceutical carriers include all of those described above. The carrier selected will depend upon the route of administration, and the form in which NrS1 is introduced. In addition, the formulations of the present invention may be prepared by methods well known in the pharmaceutical art, including all of the preparation methods described above.

[0165] The present invention is also directed to pharmaceutical compositions comprising nucleic acid encoding NrS1 and a pharmaceutically-acceptable carrier, wherein the nucleic acid

expresses NrS1 in an amount sufficient or effective to treat neural degeneration, as defined above, in a subject to whom said pharmaceutical composition is administered. Such a pharmaceutical composition would be useful for administering NrS1 to a subject in need of treatment for neural degeneration, in order to treat the neural degeneration in the subject. An amount of the nucleic acid is provided to the subject such that it expresses NrS1 protein in an amount that is effective to treat the neural degeneration, as defined above, in the subject. These amounts may be readily determined by the skilled artisan. Additionally, the pharmaceutical compositions may be administered to a subject in accordance with all of the above-described methods for administration and introduction of nucleic acids.

[0166] Formulations of these pharmaceutical compositions may be conveniently presented in unit dosage, and may be presented in a form suitable for administration of nucleic acid (*e.g.*, by injection). The nucleic acid encoding NrS1 may be presented in any form well known in the art for the introduction of nucleic acids, including, without limitation, naked DNA, plasmid DNA, and vector DNA (including viral vectors, as described above), and may be prepared in accordance with methods well known in the arts of gene therapy and molecular genetics. In addition, the pharmaceutically-acceptable carrier of the present invention must be "acceptable" in the sense of being compatible with the other ingredients of the composition, and not deleterious to the recipient thereof. Examples of acceptable pharmaceutical carriers include all of those described above. The carrier selected will depend upon the route of administration and the form in which nucleic acid encoding NrS1 is introduced. In addition, the formulations of the pharmaceutical composition of the present invention may be prepared by methods well known in the pharmaceutical art, including all of the preparation methods described above.

[0167] Some preferred embodiments of the invention are described in the following Examples. Other embodiments within the scope of the claims herein will be apparent to one skilled in the art from consideration of the specification or practice of the invention as disclosed herein. It is intended that the specification, together with the examples, be considered exemplary only, with the scope and spirit of the invention being indicated by the claims which follow the Examples.

Example 1. Molecular characteristics of neuron-astrocyte interactions.

Introduction

[0168] The establishment and maintenance of the appropriate number and type of constituent cells in the central nervous system (CNS) of the mammal is a daunting problem. Not only do the correct numbers of cells end up in the correct locations during early development; in the absence of trauma or disease, the total numbers and the ratios of cell types remain relatively constant during the longest developmental phase - adulthood - when homeostasis is established. This observation implies much about homeostasis in the CNS. For example, unlike the vast majority of cells in the body, neurons are incapable of dividing and are not replaced over a lifetime. This indicates that there are unique mechanisms which provide continuous support for these cells. In the case of astrocytes, however, the situation is more complex, as these cells are able to re-enter the cell cycle at virtually any point in their history, and do so in response to trauma and disease (15). In spite of this proliferative ability, the number of astrocytes remains largely unchanged throughout life (23, 24, 25). Moreover, it has been demonstrated that astrocytes can be maintained out of the cell cycle while they are in direct contact with the neuronal surface (11, 12, 29).

[0169] It is important to determine how mitotic quiescence is established and maintained in astrocytes, as there are major sequelae, both positive and negative, that result from astrocyte proliferation in the adult mammal. These sequelae include gliotic responses to injury, the secondary blocking of axonal migration and neuronal remodeling, collateral damage to otherwise intact tissue resulting from astrocytotic spillover, and possible spontaneous mutations and/or viral integration in astrocytes transiting the cell cycle. All of these can lead eventually to astrocytoma.

[0170] In the case of CNS injury, resulting astrocytosis is thought to be a major contributor to the formation of a glial scar, which in turn may play an important role in blocking regenerating axons (6). While the isolation of damaged tissue is likely to be an important aspect in re-establishing the blood-brain barrier, this behavior implies that such an isolated area is permanently removed from the neural tissue available for regeneration. Further, because astrocytes do proliferate throughout life, albeit at very low levels (7), they are vulnerable to errors in DNA replication, as well as viral integration. This makes these cells susceptible to transformation over the lifetime of the mammal. Indeed, in those people who have been diagnosed with a brain tumor, the majority of tumors will be of astrocyte lineage. In view of the foregoing, it is clear that an understanding of the basic biology of neuron-glia interaction may provide insight into the means by which astrocyte growth control is both initially achieved and

subsequently maintained throughout life. Such knowledge likely will yield further insight into methods for re-establishing ordered growth in transformed cells.

[0171] Earlier work has demonstrated that astrocytes become growth-arrested upon contact with the neuronal cell surface, and that this activity is neuron-specific in the CNS (28). It also has been shown that the antiproliferative activity of neurons can be isolated from the ability of neurons to drive astrocyte-process outgrowth, by treating astrocytes with either fixed neurons or a neuronal-membrane-enriched protein fraction. In the presence of these preparations, astrocytes exit the cell cycle, but fail to extend astrocytic processes (28) or to upregulate the glutamate transporter, GLT-1 (unpublished observation).

[0172] While the cell biology of neuron-astrocyte interactions has been amply described, the molecular correlates of this cell biology have been largely underexplored. In order to begin to define the nature of these interactions, the inventor has undertaken a series of differential gene screens to compare expression patterns in purified astrocyte cultures with those in astrocytes that are co-cultured with neurons. To avoid the problem of contaminating astrocyte RNA with RNA from the "effector" neurons, the inventor took advantage of his earlier observation that neuronal cell membranes are sufficient to drive astrocytes into quiescence (29).

[0173] From that observation, the inventor identified a number of genes in which expression was upregulated by the neuron-stimulated astrocyte. The majority of genes that were identified in this assay system were known, and had well-documented expression patterns in non-neural tissues. Some of the genes in the screen were known to be expressed in the nervous system, but little was known of their biological significance. Among this latter class was a tetraspanin, CD81, also known as Target of Anti-Proliferative Antibody or TAPA. The expression of CD81 had been shown in astrocytes, and was known to be upregulated following neural trauma (8). However, the function of CD81 in trauma, and in homeostasis, was previously undefined.

[0174] Using a combination of antibody perturbation, biochemical competition, and gene-knockout studies, the inventor has shown that CD81 is a critical modulator of astrocyte growth control. See, e.g., FIG. 21. This observation is crucial, as astrocytosis results from numerous neural traumas, and the resulting glial scar is believed to present a major barrier to productive neural regeneration. In addition, astrocytomas are the predominant single form of brain cancer, with a prevalence on the order of 17,000 cases per year. All astrocytoma cells tested herein failed

to express CD81 message or protein, raising the possibility that CD81 plays an important role in astrocyte-tumor formation and/or metastasis.

[0175] In addition, the inventor describes herein the identification of the neuronal ligand for CD81, which has been named NrS1. The inventor also discloses a series of events that ensue from stimulation of neurons with a small mimetic of CD81, which has been named GM1109.

Materials and Methods

[0176] Animals. Pregnant Sprague-Dawley rats and C57BL/6 mice were obtained from Charles Rivers Laboratories. CD81 heterozygous mice were backcrossed greater than 10 generations into the C57BL/6 background. The generation of these mice has been previously described (17). Heterozygous animals were crossed, and offspring were born with the expected Mendelian frequency. However, an attenuated postnatal viability was observed in the CD81-/- animals. The genotypes of the progeny of these crosses was determined exactly as described (17). Notably, in earlier backcrosses, there was normal Mendelian distribution and normal survival of embryos, but death common in the perinatal period in the homozygous null animals.

[0177] Tissue Culture – Primary Neural Cells. Primary cerebellar neurons and astrocytes were prepared as described (18). In brief, cerebella were dissected from rat or mouse pups at postnatal day 4 or 5, the meninges were stripped, and the remaining tissue was washed in Ca^{2+}/ Mg^{2+} free PBS (CMF-PBS). The tissue then was trypsinized, triturated through decreasing caliber needles in the presence of DNase, and pelleted. The cells were resuspended in CMF-PBS, and the single cell suspension was overlaid and separated on a Percoll step gradient (30/60%; Amersham Pharmacia), all as described (18). Following extensive washing to remove residual Percoll, the neuron- and astrocyte-enriched fractions were further enriched: differential adhesion removed contaminating astrocytes from the neuron preparation, and treatment with anti-Thy1 and complement-mediated cytolysis removed neurons and fibroblasts from the astrocyte-enriched fraction. All cells were cultured in D^{10}, consisting of DMEM (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gemini-Bio-Products, Inc.), 10% heat-inactivated horse serum (Gemini-Bio-Products, Inc.), 1% non-essential amino acids (Gibco), penicillin-streptomycin (Gibco; 20 U/ml), Fungizone (Gibco; 0.25 μg/ml), and glucose at 0.6% final concentration. The astrocyte cell suspensions were seeded at 2 x 10^5 cells/well in 24-well plates (Costar) or 5 x 10^4 cells/well in 8-well Lab-Tek tissue culture chambers (Nalge Nunc), which had been treated with

50 μg/ml poly-L-lysine (Sigma). Effector neurons were added at a ratio of 2 neurons per astrocyte.

[0178] Astrocytoma cell lines. Several astroglial cell lines (rat C6 and 9L, human A172 and U251MG, and mouse LN308 and LN18) were grown in 100-mm tissue culture dishes (Falcon Labware) in D^{10}.

[0179] Antibodies. Rabbit anti-cow glial fibrillary acidic protein (GFAP) antibodies, as well as TRITC-conjugated swine anti-rabbit antibodies, were obtained from DAKO A/S (Copenhagen, Denmark). Mouse monoclonal antibodies (mAbs) to bromodeoxyuridine (BrdU) and conjugated to FITC were obtained from Boehringer (Mannheim, Germany). Hamster mAb 2F7 against CD81 (1) and FITC-conjugated mouse anti-hamster mAbs were obtained from Pharmingen (San Diego, CA). Hamster mAbs Eat1 and Eat2 react with distinct epitopes in CD81, and have been recently described (18). TuJ1 recognizes a neuron-specific βIII subunit of tubulin. Alexa red conjugated goat anti-mouse secondary antibody was purchased from Molecular Probes, and mouse mAb anti-GST was purchased from Sigma. Biotinylated goat anti-mouse antibody and the Vectastain ABC kit were purchased from Vector Labs.

[0180] Fusion Proteins. Full-length SCIP and the region of the mouse CD81 encoding the large extracellular loop (LEL) were both cloned into pGEX expression vectors (5), to generate GST fusion proteins. Clones were sequenced, and DH5α *E. coli* were transformed with the respective clones and IPTG induced. The resulting lysate was enriched on glutathione-agarose beads, and the protein concentration was assessed by BCA assay (Pierce). The integrity of the material was determined by gel electrophoresis and immunoblotting with antigen-specific antibodies and/or anti-GST antibodies, all by standard techniques.

[0181] Northern Blot Analysis. Total RNA was extracted from cultured cells, as described by Chomczynski and Sacchi (2). 20 μg of RNA from each sample were electrophoretically separated on denaturing agarose gels, and transferred to nylon membranes (Micron Separations Inc.). The membranes were probed overnight at 42°C with random primed, [^{32}P]dCTP-labeled mouse CD81 cDNA, washed sequentially (three times for 15 min at 65°C in 2x SSC, 0.1% SDS; twice for 10 min at 65°C in 0.2x SSC, 0.1% SDS; and once for 5 min at room temperature in 2 x SSC), dried, and exposed to X-ray film.

[0182] Immunoblotting. Cultured cerebellar astrocytes and astrocytes co-cultured with neurons C6 astroglioma were washed twice in ice-cold PBS, scraped from the dishes, pelleted, and resuspended in hypotonic disruption buffer (10 mM HEPES (pH 7.9), 10 mM NaCl, 0.1 mM

EGTA, 0.1 mM EDTA, 0.5 mM phenylmethylsulfonylfluoride, 0.5 mg/ml leupeptin, 0.7 mg/ml pepstatin A, and 1 mg/ml aprotinin). The samples were incubated on ice for 15 min, after which NP-40 was added to a final concentration of 1%. The detergent-soluble and -insoluble portions were separated by centrifugation. Protein concentrations were determined for the membrane-containing detergent-soluble fraction using a BCA assay (Pierce). 50 μg of protein were separated on a 10% SDS-polyacrylamide gel, and the proteins then were transferred onto nitrocellulose using a semidry blotter. The efficiency of transfer was determined by amido black staining. The membrane was blocked in Buffer A, containing 5% milk and 1% Triton-X 100 in Tris buffered saline. The membrane was then probed with goat anti-CD81 antiserum, followed by peroxidase-conjugated donkey anti-goat secondary antibody. The reaction product was visualized by ECL.

[0183] Immunofluorescence. Forty-eight hours after plating, cell cultures were washed in PBS and fixed in 4% paraformaldehyde in PBS at 4^{O}C for 30 min. Nonspecific binding was blocked by incubation in 10% FBS/PBS for 30 min at room temperature. The blocking solution was removed, and primary antibodies (2F7, Eat1, or Eat2 diluted in PBS) were added at 37^{O}C for 1 h. The cultures were rinsed, incubated in mouse FITC-conjugated anti-hamster antibodies at room temperature for 30 min, washed, and mounted (Pro Long Antifade Kit, Molecular Probes). The same types of cultures were also double-labeled for GFAP and BrdU incorporation with FITC-conjugated anti-BrdU antibodies (Sigma) (see below). The cultures were washed in PBS, and fixed in 4% paraformaldehyde/PBS at 4^{O}C for 30 min, washed in PBS, and permeabilized in 0.5% Triton X-100/PBS at room temperature for 10 min. Nonspecific binding was blocked by incubation in 10% FBS/PBS at room temperature for 30 min, and GFAP was visualized with a TRITC-conjugated secondary antibody.

[0184] *In vitro* proliferation assays. Cultures were established, either under control conditions, or in the presence of fusion protein or increasing concentrations of one of the mAbs as described herein. Twenty-four hours later, 10 μM BrdU (Sigma) was added, and the cultures were continued for an additional 24 h. Subsequently, the cells were fixed, and astrocytes were identified by GFAP staining, as described herein. To visualize BrdU incorporation, the chromatin was denatured in 2 M HCl for 30 min, washed extensively in PBS, and incubated with FITC-anti-BrdU antibodies at room temperature for 1 h. Following incubation, the cells were washed extensively, stained with *bis*-benzamide (Sigma) to determine total cell numbers, washed again, and mounted in Anti-Fade. The level of astrocyte proliferation was determined by dividing the

number of BrdU-positive astrocytes by the total number of GFAP-positive cells per microscopic area. All proliferation assays were repeated at least three times; 30 microscopic areas were examined from each experimental sample (600-700 cells/sample, for a minimum total of ~2000 cells per experimental point). Statistical analysis of the data was performed using two-tailed, students' *t*-test.

Results

[0185] CD81 is expressed on the cell surface of the astrocyte. The inventor originally identified CD81 expression in astrocytes co-cultured with neuronal membranes by using a differential screening approach. To determine if CD81 protein was indeed expressed by astrocytes, the inventor established cultures of astrocytes or the astrocytoma cell line, C6, as previously described (28). Protein from 48-hour cultures was isolated, separated on an SDS-PAGE gel, and blotted with a polyclonal antibody against CD81. CD81 is constitutively expressed by cultured astrocytes, whereas the C6 glioma cells are CD81-negative (FIG. 1A). The addition of neurons to these cultures increased CD81 expression by 50-70% in astrocytes, but had no effect on the C6 cells (some data not shown). To localize CD81 expression on the astrocytes, an anti-CD81 monoclonal antibody (mAb), 2F7 (1), was used to stain rat astrocytes cultured alone, as well as co-cultures of astrocytes and neurons. Figure 1B shows the punctate staining pattern of CD81 on the surface of astrocytes. When co-cultured with neurons, astrocytes extend complex processes that serve as guidance pathways for neuronal migration, and matrices for neuronal adhesion and differentiation (11, 29). Staining of neuron-astrocyte co-cultures showed a punctate pattern of CD81 expression, both on the astrocyte cell soma and along the processes (FIG. 1C, arrows). In contrast, neurons in these cultures failed to stain with the 2F7 antibody.

[0186] Anti-CD81 mAbs recognize unique, non-overlapping, extracellular epitopes. CD81 has four transmembrane domains, resulting in two loops in the extracellular domain. One is the small extracellular loop (SEL), and the other is the large extracellular loop (LEL). When the 2F7 antibody was used on live, non-permeabilized astrocytes, results indicated that it recognizes an extracellular epitope (FIGS. 1A and 1B). Recent work has shown that the 2F7 mAb recognizes a conformationally-dependent epitope that requires the presence of both the SEL and LEL of CD81. While Eat2 has a higher affinity for CD81 than does 2F7, it also requires both loops for antigen binding. In contrast, Eat1 recognizes an epitope within the LEL (18). In an

effort to determine if any of these mAbs was able to block neuron-astrocyte interactions, the inventor established co-cultures in the presence of these reagents.

[0187] Neuron-astrocyte interactions are blocked *in vitro* by mAb Eat1. In order to determine the potential efficacy of Eat1 and Eat2 in blocking neuron-induced astrocyte differentiation and cell-cycle exit, the inventor established co-cultures of cerebellar granule cells and astrocytes. The cultures were allowed to grow for 48 h, with BrdU added in the last 24 h of culture. As can be seen in FIG. 2A, there is a loss of neuron-induced astrocyte growth arrest, which is dependent on the concentration of the Eat1 antibody. Notably, the neurons in this culture were viable, and adhered to the astrocytes and extended neurites (see below). Previous work has shown that cerebellar granule cells are exquisitely dependent upon astrocytes and astrocyte-derived factors for survival (13). The inventor's observation that the Eat1 mAb blocked neuron-dependent astrocyte proliferative arrest, but not trophic support, suggests that all normal neuron-astrocyte interactions are not lost under these conditions.

[0188] Eat2 had no apparent effect on neuron-astrocyte interactions: neuron-astrocyte co-cultures established in the presence of Eat2 were indistinguishable from control co-cultures. Under both control and Eat2 conditions, astrocytes withdrew from the cell cycle, and extended complex processes, when challenged with neurons (FIGS. 2D and 2E). In contrast, the addition of mAb 2F7 enhanced neuron-induced astrocyte proliferative arrest (FIG. 2B), suggesting that the addition of this antibody to the co-culture system augmented neuron-dependent astrocyte growth arrest. Taken with the Eat1 data, these observations show that alterations in CD81 bioavailability and/or conformation have a profound effect on modulating astrocytic proliferative responses to neurons.

[0189] It has been previously shown that astrocyte proliferation and process formation in response to neuronal contact are separable events. Contact with neuronal membranes is sufficient for astrocyte growth control, but viable neurons are required for both cell-cycle exit and process formation (29, 30). While the inventor saw no differences in astrocytic process outgrowth in neuron-astrocyte co-cultures under control conditions or in the presence of either mAb 2F7 or Eat2, there was a remarkable difference between those cultures and co-cultures established in the presence of Eat1. Examples of these stark differences can be seen in FIGS. 2C and 2D, in which neuron-astrocyte co-cultures were set up in the presence of Eat1 and Eat2, respectively.

[0190] In the presence of Eat1 (FIG. 2C), the astrocytes failed to extend typical processes in response to neurons, and remained in the cell-cycle. The astrocytes depicted in FIG. 2C were

imaged with anti-GFAP antiserum. These have the appearance of a cluster of daughter cells that arose *in situ*. In contrast, in the presence of Eat2 (FIG. 2D), the GFAP-expressing astrocytic processes are long, and of a complexity that is indistinguishable from astrocytic responses seen in co-cultures of astrocytes and viable, wild-type granule cell neurons (FIG. 2E). In addition, the integrity of neuritic processes is uncompromised in the presence of the anti-CD81 mAbs Eat1 and 2F7 (FIGS. 2F, 2G, and 2H). These data demonstrate that the effects of the anti-CD81 mAbs occur at the level of the astrocyte, and are not the result of a reduction in viability, or attenuation of axonogenic capabilities, of the neuron. Moreover, the data show that the treated astrocytes are able to support neuronal survival and axonal outgrowth, further highlighting the specificity of the role of CD81 in one aspect of astrocyte interactions with cognate neurons.

[0191] Soluble GST-CD81 fusion protein binds to the neuronal cell surface and competes for astrocyte-expressed CD81. The antibody blocking studies were suggestive of a significant role of CD81 in mediating neuron-astrocyte interactions. However, as with any antibody blocking experiments, there is always a concern about steric inhibition. Therefore, to further extend these observations, the inventor used a soluble mouse GST-CD81 large extracellular loop (GST-CD81(LEL)) fusion protein in an effort to compete for neuronal binding to the astrocyte. To determine if the fusion protein was able to bind to neurons, astrocytes, or both, the inventor isolated and purified the respective cell types, as described (28). The viable cells were incubated with 10 μg/ml of GST-CD81(LEL) on ice for 1 h. Thereafter, the cells were fixed, then stained with an anti-GST antibody to avoid staining endogenous CD81. The GST-CD81(LEL) fusion protein adhered to the neuronal fraction, but not the astrocytic fraction (FIG. 3). The few stained cells seen in the astrocyte-enriched fraction were probably neurons, based on the size of the cell somata, and the shape of the cells. Astrocytes in culture become flat, and bipolar or tripolar, unlike these cells.

[0192] CD81 is required for normal neuronal function and neuronal/astrocyte interactions. In cerebelli of CD81 -/- mice, neuronal atrophy and abnormal histology is evident (FIG. 22). Moreover, CD81 is required for initial astrocyte/neuron interactions (FIG. 24).

[0193] The observation that the CD81(LEL) protein adhered to the surface of the neuron suggests the existence of a CD81 receptor on these cells. This putative receptor may be potentially involved in normal neuron-astrocyte interactions. To determine if blocking such a receptor would block the ability of the neuron to bind to CD81 and to differentiate the astrocyte *via* CD81, the inventor added increasing concentrations of either GST-CD81(LEL) protein or an

irrelevant GST fusion protein, GST-SCIP. The soluble CD81 blocked normal neuron-induced astrocyte proliferative arrest in a dose-dependent manner, while the GST-SCIP had no effect (FIG. 4). Neither fusion protein had any visible effects on neuronal survival or differentiation. Based on the neuronal binding patterns, as well as the blocking, these data suggest that the soluble CD81 is competing for receptors on the neuron, thereby blocking normal neuron-induced, CD81-mediated proliferative arrest (FIG. 4).

[0194] Astrocyte cell-cycle withdrawal is CD81-dependent. In addition to its expression on the astrocyte, CD81 is also expressed by numerous cell types, including lymphocytes. In the immune system, CD81 has been shown to play a vital role, as evidenced by the impaired immunity observed in CD81-deficient mice (16, 17, 20, 27). Heterozygotic CD81 mice were backcrossed 10 generations onto a C57BL/6 background, to establish the CD81 deletion in a pure C57BL/6 genotype. Mixed neuron-astrocyte cultures were established from animals immediately after birth. These CD81-/- animals were harvested in early postnatal life because they have severely decreased viability beyond the first hours of birth. At the time of harvesting, the additional neural tissue was taken for simultaneous genotyping. The cultures were established, and allowed to grow for 48 h. BrdU was added in the final 24 h of culture. The cells then were fixed and stained, and astrocyte proliferation was determined by BrdU and GFAP double labeling. The proliferation data was tabulated before the genotype of the respective cultures was unblinded. The extent of astrocyte proliferation in the wild-type co-culture was set at 1. With respect to this level of proliferation, CD81+/- animals showed a 20% increase in astrocyte BrdU incorporation, whereas the CD81-/- astrocytes showed a doubling of astrocyte proliferation (FIG. 5).

[0195] CD81 is absent in a variety of astrocytic tumor cell lines. Tumorigenesis is a multistep phenomenon which contributes to a loss of growth control. To determine if CD81 might play a role in either astrocytic tumor progression or metastasis, the inventor assayed a number of astrocytoma cell lines for CD81 mRNA expression. Consistent with the immunofluorescence data, astrocytes expressed CD81 mRNA when isolated and cultured as a purified cell population. Upon co-culture with neuronal membranes, astrocyte expression of CD81 was upregulated, suggesting a positive feedback mechanism in maintaining astrocytes out of the cell cycle. In contrast, none of the astrocytoma cell lines assayed had detectable levels of CD81 message after 3 days of exposure (FIG. 6). Lack of CD81 in astrocytomas can also be demonstrated by immunohistochemistry (FIG. 23). Furthermore, astrocytoma cells in which CD81 is re-expressed revert to normal appearance and function (FIG. 25).

[0196] There are likely to be a number of layers of neuron-regulated growth control, as no gross astrocytosis was observed in the CD81-/- animals when they were in the C57BL/6 background. However, when CD81-/- mice are bred into a BALB/c background, there is massive astrocytosis (8, and personal communication). Thus, there may be additional genetic components that interact with CD81 to regulate astrocyte proliferation *in vivo*. Nevertheless, the present data suggest that CD81 may play a role in astrocyte tumor progression.

[0197] NrS1 binds CD81. The specificity of neuronal-membrane binding to astrocytes is demonstrated in FIG. 9, which shows the saturable binding of ^{35}S-labeled granule cell neuron protein to monolayers of astrocytes (closed diamonds). In contrast, PC12 cell membrane protein, prepared in the same manner, failed to demonstrate any significant binding to astrocytes. Neither protein fraction showed any saturable binding to monolayers of 3T3 cells (data not shown).

[0198] The saturable nature of neuronal-protein binding to astrocytes, as well as the ability of neuronal protein to induce astrocyte growth arrest, led the inventor to devise a protein purification strategy to isolate the active agent. The inventor's strategy included the capture of membrane-associated proteins using Triton-X 114 (FIG. 10A, lane 1) or FPLC chromatography on an anion-exchange column (FIG. 10A, lane 2), followed by affinity chromatography using astrocyte membrane affinity matrix and eluting with either EDTA (FIG. 10A, lane 3) or NaCl (FIG. 10A, lane 4). The predominant species in the NaCl-eluted fraction was a diffuse band at 70 kD (see below). The material was assayed at every step. A 10,000-fold enrichment in activity was obtained in the salt-eluted fraction. In contrast, there was a lack of antiproliferative activity in the EDTA-eluted fraction. This suggests that the neuron-expressed antve proteins bind independently of divalent cations.

[0199] The inventor also developed an assay to determine activity of the various protein preparations. In this assay system, proteins of interest can be bound to a clear matrix; in turn, cells can be plated onto the protein-matrix substratum and assayed for a variety of activities, including gene expression. With this assay, the inventor was able to remove bound proteins from the enrichment procedure described above, and assay them for astrocyte proliferation. In brief, following adsorption of the test proteins to the matrix, purified mouse astrocytes were added for 48 h, and the number of astrocytes was calculated.

[0200] In addition, the inventor assayed for the subcellular distribution of the protein, statin – a 57-kD protein that has been shown to translocate from the cytosol to the nucleus in cells that are in G_0 (39, 40, 41). FIG. 10B demonstrates that astrocytes, when plated on the NaCl-

eluted fraction, mobilize statin to the nucleus from the cytosol. This activity is quantified in Table 1. After identifying the astrocyte receptor for this activity, the inventor was able to positively identify the neuronal ligand, which has been named NrS1. The cloning and characterization of the NrS1 receptor on astrocytes (*i.e.*, CD81) and the full identification of NrS1, are described herein.

Table 1. Neurons induce astrocytes into G_0.

	Control	EDTA Eluate	NaCl Eluate
Cells per fields	59 ± 9.6	53.1 ± 8	9.4 ± 2.3
Inhibition of proliferation	0%	10%	100%
Statin$^+$ nuclei per field	0%	4%	100%

[0201] GM1109 binds to the neuronal cell surface, competing for astrocyte-expressed CD81. Using antibodies, the inventor previously showed that the disulfide-bonded side-pocket of the large extracellular domain (ECD) of CD81 is required for the transduction of neuron-induced astrocyte growth arrest (34). To further probe this putative binding pocket, the inventor generated GM1109, a mimetic based on modeling predictions. GM1109 is a peptide compound designed to fit the neuronal binding site of CD81. The inventor engineered the peptide as a fusion protein with the glutathione-binding domain of glutathione s-transferase. The fusion protein was used for the purpose of affinity purification, and as a means of following peptide binding and function.

[0202] The inventor's modeling predicted that GM1109 would be able to compete with NrS1 for binding to CD81. To test this prediction, the inventor added increasing concentrations of either GM1109 protein or an irrelevant peptide, GM1110, to co-cultures of neurons and astrocytes. Consistent with the inventor's model, GM1109 blocked neuron-induced astrocyte proliferative arrest in a dose-dependent manner, while GM1110 had no effect (FIG. 11). Neither compound had any observable effect on neuronal survival or differentiation (data not shown), thereby suggesting that the effects are at the level of signaling, not neuronal survival.

[0203] There are two potential interpretations of these data: (1) GM1109 interacts with (an) unknown protein(s) in *cis* on the astrocyte cell surface, preventing the protein(s) from participating in NrS1 signaling through CD81; or (2) GM1109 binds in *trans* to NrS1 on the surface of the neuron, thereby antagonizing signaling. To study these possibilities, the inventor isolated and purified the two cell types, as previously described (27). In brief, cells were acutely isolated from neonatal rodent cerebellum, and separated into neuron and astrocyte fractions by density centrifugation and differential adhesion. Identical numbers of viable neurons or astrocytes were incubated with 10 μg/ml of GM1109 on ice for 1 h. The cells were fixed, and then stained with an anti-GST antibody to avoid staining endogenous CD81. As shown in FIG. 12, GM1109 binds exclusively to the neurons, confirming that this molecule acts in *trans* and binds the neuronal cell surface.

[0204] GM1109 binds to a single, neuron-specific protein. As described above, it appeared that at least one neuronal membrane protein is required for the induction of astrocyte growth arrest. In light of this observation, the inventor reasoned that GM1109 might be used as a hook to capture and identify the neuronal protein of interest, because it is able to bind the neuronal cell surface. In brief, the inventor purified granule cell neurons from early postnatal mouse and rat, and generated detergent-soluble extracts from these cells and from rat Schwann cells and 3T3 fibroblasts. The proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with GM1109, followed by goat anti-GST. Under these stringent conditions, GM1109 protein interacted specifically with a ~70-kD neuronal protein, which was absent from the control cells (FIG. 13A). To date, with the exception of central neurons, no other cell type is known to express this protein.

[0205] The CD81 ligand is a transmembrane tyrosine kinase. Based on the cell-surface binding of GM1109 (see FIG. 12), the inventor used GM1109 to first bind to, and then pull down, its cognate ligand. Briefly, the inventor bound GM1109 to purified granule cell neurons on ice for 10 min. These cells then were lysed in the presence of protease inhibitors, and the GM1109-ligand complexes were purified on glutathione/agarose beads. Following extensive washing, the protein complexes were eluted from the beads, and separated by SDS-PAGE. Control cells (3T3 cells) were run in parallel. The gel then was silver-stained to reveal the isolated proteins (FIG. 14). The only protein associated with GM1109 was approximately 70 kD, and was not expressed by the control cells.

[0206] Using the same procedure, the inventor isolated the p70 band, and subjected it to Edman degradation and mass-spectrometry analysis. Based on these analyses, GM1109 apparently binds to a protein tyrosine kinase, referred to herein as NrS1. It is not surprising that, at the structural level, NrS1 has a high level of homology with a number of other tyrosine kinases in the enzyme domain. However, the NH_2 terminus, which is modeled as the extracellular domain of NrS1, is unique.

[0207] Many tyrosine kinases are themselves targets of phosphorylation. In order to test if NrS1 is itself phosphorylated in response to GM1109 binding, the inventor conducted a time course of binding of GM1109 to freshly-isolated human neurons, which were purified from late-term abortus. Following isolation and purification, the cells were mixed with GM1109 for 0, 2, or 10 min. Thereafter, the cells were lysed, and the GM1109-NrS1 complexes were purified. Following separation by SDS-PAGE, the material was transblotted to nitrocellulose, and probed

with an anti-phosphotyrosine antibody. Within 2 min, NrS1 was fully tyrosine-phosphorylated; by 10 min, the signal was quenched, suggesting that binding of NrS1 to the CD81 agonist initiates a neuronal-signaling cascade (FIG. 15).

[0208] Full astrocyte differentiation depends upon bi-directional signaling. The inventor previously demonstrated that contact with neuronal membranes is necessary for astrocyte growth arrest, but is not sufficient to drive astrocyte-process formation or the upregulation of glutamate transporters (28, and unpublished data). In contrast, viable neurons effect both astrocyte quiescence and full differentiation. The requirement for viable neurons to initiate and drive full astrocytic differentiation suggests that, following CD81 signaling, neuronal responses include changes in gene expression that, in turn, effect the astrocytic responses. The inventor's observation that CD81 induces a rapid phosphorylation of NrS1, and is therefore likely to initiate a signaling cascade in the neuron, is consistent with this hypothesis.

[0209] To begin to investigate this system, the inventor established cultures of astrocytes with neuronal membranes, plus or minus dibutyryl-cAMP (dbcAMP), as a mimetic of post-viable neuron signaling events in the astrocyte. This system has been used by others to model astrocytic responses to intact neurons (37, 38, 44). dbcAMP had no effect on the morphology of astrocytes grown in the absence of neurons (FIG. 16A). The inventor previously has demonstrated that dbcAMP has no effect on the astrocyte proliferative index (42). The addition of neuronal membranes to cultured astrocytes arrested astrocyte proliferation, but failed to induce process outgrowth (FIG. 16B). However, the addition of neuronal membranes plus dbcAMP allowed for both growth arrest and process formation (FIG. 16C).

[0210] Previous work has shown that astrocyte-derived soluble factors support neuronal survival (33). Here the inventor has begun to define a potential mechanism by which astrocytes signal to neurons, *via* CD81, thereby setting off a cascade, through phosphorylated NrS1, in which the neuron signals back to the astrocyte.

[0211] Binding of a CD81 agonist, GM1109, to NrS1 induces neuronal survival and paracrine signaling to astrocytes. It has been appreciated for decades that neurons and glia are interdependent for survival and differentiation; however, the molecular mechanisms underlying these events are only partially understood. One such mechanism that has been partially elucidated is the neuron-induced differentiation of glia by neuregulin (Nrg) (43). Nrg on CNS neurons has been shown to influence astrocyte differentiation (36) by signaling *via* Nrg cognate receptors on astrocyte erbB2 and erbB4 (31), resulting in increases in astrocyte cAMP levels (32). These data

fit the inventor's observations that full astrocyte differentiation is dependent upon both CD81-NrS1 signaling and elevation of astrocyte cAMP levels.

[0212] To explore whether CD81-NrS1 interactions mediate downstream Nrg signaling in neuron-astrocyte interactions, the inventor purified granule cell neurons and treated the GM1109, as a mimetic for astrocyte stimulation. Such treatment induced profound and rapid changes in the neuron, as described below, and suggests that GM1109 might have powerful properties that would be useful in the treatment of neurodegenerative conditions.

[0213] Granule cell neurons express a transmembrane form of neuregulin, which is cleaved upon CD81 signaling. Granule cell neurons were purified to >99% homogeneity, as described above. The cells were cultured in defined medium, with no added growth factors, and then stained with an antibody that is specific for the intracellular domain of neuregulin (ICD_{Nrg}) (35). In FIG. 17A, the staining pattern shows that the ICD_{Nrg} was associated with the cell membrane in these acutely-dissociated neurons. However, when these cells were treated for as little as 5 min, all of the ICD_{Nrg} was lost from the cell surface. Moreover, the ICD_{Nrg} migrated to the neuronal nucleus (FIG. 17B), where it is believed to mediate transcriptional changes that allow for neuronal survival and neurite outgrowth (see below). In addition, treatment with GM1109 induced a 2-fold increase in Nrg concentration in the supernatant of cultured granule cell neurons (FIG. 17D).

[0214] Taken together, these data suggest that shortly after the astrocyte mimetic, GM1109, binds to neurons, there is a cleavage of the transmembrane form of Nrg, the extracellular astrocyte-signaling domain is released, and the ICD_{Nrg}, which has a nuclear localization signal, moves to the neuronal nucleus. Such a biology would explain the mechanism by which neurons, via NrS1, induce astrocyte growth arrest, and then induce full maturation via Nrg signaling. The inventor has termed the reactions in which neurons signal to astrocytes as "forward signaling", and the resulting intracellular neuronal signaling events as "reverse signaling".

[0215] GM1109-induced reverse signaling rescues neurons and induces process outgrowth. Neuronal survival has been shown to be dependent upon astrocyte-derived signals (33). However, the precise manner in which astrocytes signal to neurons has not been well documented. In order to determine if the CD81 mimetic, GM1109, is sufficient to initiate a cascade of neuronal survival and axonogenesis, the inventor cultured purified granule cell neurons at low density (10^5 cell/well in a 24-well plate), and added either GM1109 or an irrelevant control peptide.. The cells were assessed daily for survival. After 96 h, the cells were fixed and double stained for both neurons and astrocytes. Not unexpectedly, the cells were >99% neurons, with a

few, isolated GFAP-positive cells. Neurons cultured in the presence of the astrocyte-signaling mimetic, GM1109, displayed tremendous survival advantages over cells cultured under control conditions, with few pyknotic cells, and numerous process-bearing cells. In contrast, under control conditions, few cells survived, and those that did survive had limited neurite outgrowth.

[0216] At the conclusion of the experiment, the cells were imaged by indirect immunofluorescence. An example is seen in FIG. 19. Cells cultured in the presence of GM1109 had large, complex neurites (FIGS. 19B and 19C). In contrast, in control cultures, there were few viable cells, and only a few short neurites on those cells that survived (FIG. 19A). Live/dead staining, 24 h after explantation, revealed a greater-than-15-fold increase in viability of neurons that had been cultured with GM1109, as compared to control-treated cultures.

[0217] Fifteen amino acids of GM1109 rescue CNS neurons from cell death. In an effort to further define the domain(s) of GM1109 that signal(s) to neurons, and induce(s) or enhance(s) neuronal survival, the inventor took advantage of data previously showing that a monoclonal antibody, Eat1, blocks neuron-induced astrocyte growth arrest (34). Epitope-mapping of the Eat1 antigen defined a region in the large ECD of CD81, thereby suggesting that the function of peptides corresponding to this region might be sufficient. To test this possibility, the inventor synthesized three overlapping 20-mers. The C-terminal 5 amino acids of each peptide overlapped with the N-terminal 5 amino acids of each succeeding peptide. The inventor generated three such peptides, corresponding to a total of 60 amino acids of the large ECD of CD81. The peptides were purified by HPLC, yielding >99% purity. A dose curve of each peptide was added to cultures of purified granule cell neurons, as was GM1109 (as a positive control). The cells were maintained in Sato's defined medium, that was supplemented with 0.5% fetal calf serum, for a total of 5 days (120 h). Thereafter, viability was determined with a commercial live/dead stain (Molecular Probes). This assay system is advantageous because esterases in viable cells cleave the calcein dye. The cleavage product then fills the cells, revealing the extent of neurite outgrowth, and permitting the easy identification of neurons.

[0218] As shown in FIG. 20A, in the absence of any treatment, virtually all of the granule cell neurons died, while the positive control, GM1109, rescued the vast majority of cells, in a manner consistent with the data shown in FIG. 19. Of the peptides tested, GM1415 had no activity in this assay. In contrast, GM1416 had potent activity in rescuing neurons from cell death, supporting the possibility that GM1416 might be a novel therapeutic for salvaging damaged neurons and enhancing the survival of neurons.

Discussion

[0219] The establishment of the proper ratio of cell types within the mature CNS is not fully understood. While most neuronal populations are unable to re-enter the cell cycle after cellular differentiation, the same does not hold true for astrocytes. These cells are able to proliferate at any point in the life of the mammal, and do so under a variety of pathological conditions. However, in homeostasis, the number of astrocytes is remarkably conserved and maintained at a steady state (23, 24, 25). Previous work has shown that neuronal cells are a potent effector of astrocytic proliferative arrest and terminal differentiation. Moreover, these same mechanisms are likely to keep the astrocyte out of the cell cycle throughout life. While numerous candidate molecules have been proposed to be mediators of this activity, including NCAM (9), atrial natriuritic protein (21), astrotactin (4, 22), and endothelin 1 (26), none has withstood rigorous analysis.

[0220] Much of the analysis of neuron-astrocyte interactions has been modeled *in vitro*. When challenged with neurons under culture conditions, astrocytes withdraw from the cell cycle, and extend complex, GFAP-rich processes (11, 12, 29, 30). Herein, the inventor reports his recent findings that CD81 is a critical modulator of neuronal-mediated astrocyte differentiation and proliferative arrest. This conclusion is based on three separate, independent lines of experimental evidence. Antibody blocking, antigen competition, and genetic approaches all converge to suggest that CD81 is a critical part of this biology. Moreover, astrocytic tumor cell lines which were tested are CD81 deficient, further suggesting that CD81 is likely to play an important role in normal neuron-astrocyte biology. Importantly, the *in vitro* findings phenocopy *in vivo* events in some genetic backgrounds but not others, implying that CD81 either acts as a modifier of, or is modified by, additional genes.

[0221] Function-blocking antibodies are valuable tools for testing critical molecular interactions. The inventor has shown herein that Eat1, which binds to a discrete epitope located in the LEL of CD81, is able to ablate astrocytic responsiveness to co-culture with neurons, *i.e.*, the astrocytes remain in the cell cycle, and fail to fully differentiate. In these studies, neurons still were able to adhere to the astrocytic cell surface, where they settled and extended prototypical, complex processes. The survival and differentiation of the neuronal cells suggest that there are multiple layers for neuron-astrocyte interactions, and that the astrocytes in these cultures were able to maintain the health of the neurons, even without full differentiation of the astrocytes. The

requirement for astrocytes, or astrocyte-derived support, for granule cell survival and differentiation is well known (13). Therefore, these data suggest that CD81 activity in neuron-astrocyte interactions is specific to neuron-induced astrocyte differentiation.

[0222] Conformational changes, induced by binding of the various anti-CD81 mAbs to their cognate antigens, result in distinct astrocytic responses to neurons. While Eat2, which binds avidly to CD81, has no effect on function, the Eat1 antibody blocks interactions between CD81 and a heretofore unidentified partner. The idea of molecular cross-talk between CD81 and an unknown partner is supported by the observation that 2F7 increases the sensitivity of astrocytes to neuronal antiproliferative signaling, suggesting that conformational changes in CD81 may have profound effects on its activity. This idea is further supported by evidence that shows that the 2F7 mAb is able to block thymocyte maturation (1). There has been at least one other tetraspanin, the Drosophila *late bloomer* gene, known to have a role in recognition between neural elements in development. Flies mutant in the *late bloomer* locus fail to make proper neuromuscular synapses in a timely fashion, suggesting a role in recognition of cellular elements in the fly nervous system (14). The tetraspanins, of which CD81 is a member, are thought to be molecular facilitators, bringing together partners within the plane of the membrane (19). A definitive answer to the molecular mechanism of CD81-mediated signaling between the astrocyte and the neuron awaits the identification of an astrocyte-expressed CD81 binding partner.

[0223] In general, studies using function-blocking antibodies are inherently limiting, because there are issues of non-specific steric inhibition for which it is difficult to control. The inventor has addressed this potential problem by competing for CD81 binding using soluble, GST-CD81(LEL) fusion proteins. In this assay, the GST-CD81(LEL) fusion proteins bind to the cell surface of neurons, but not astrocytes. The binding of neurons over astrocytes, and the failure of an irrelevant fusion protein to either bind or block function, suggests a specificity of binding, and raises the likelihood of a neuron-expressed CD81 receptor. More importantly, by competing with astrocyte-expressed CD81 for the putative neuronal CD81 receptors, these soluble GST-CD81(LEL) proteins block neuron-induced astrocytic responses. These observations provide direct evidence that CD81 plays an important role in establishing neuron-induced astrocyte activity.

[0224] The final confirmation that CD81 plays a vital role in neuron-astrocyte biology was provided by establishing cultures from CD81 heterozygous and homozygous null mice. Using genetics to reduce or ablate CD81 expression, the inventor demonstrated a strict requirement for

CD81 in neuron-induced astrocytic responses. The CD81 mice used in these studies were thoroughly backcrossed onto a C57BL/6 background. The +/- mice did not develop spontaneous astrocytic tumors, nor did they show signs of astrocytic hyperplasia, astrocytosis, or any detectable neurological abnormality. However, when the CD81 deletion was backcrossed onto the BALB/c background, a profound astrocytic hyperplasia resulted (8, and personal communication). This is a critical observation, as it demonstrates that there are modifiers of CD81 activity, which depend upon a genetic background to have an observable phenotype. It is notable that the association of a given gene product needs to be considered in the light of the surrounding genome, and not *en vacuo*.

[0225] The implications of the current study extend beyond questions of regulating astrocyte cell number in homeostasis and injury. The inventor has examined a number of astrocytic tumor cell lines, all of which have severely attenuated levels of CD81 expression. While there is no evidence to suggest that CD81 is a classical tumor-suppressor gene, the absence of CD81 in these astrocytoma cell lines, taken together with CD81 function in normal neuron-astrocyte biology, raises the possibility that CD81 may be part of a tumor-suppressor cascade. The data presented here raise the possibility that mechanisms aimed at re-expressing CD81 in astrocytic tumors *in situ* may be of significant benefit for patients suffering from astrocytomas. Such an approach would be intended to limit the proliferative rate of the tumor cells *in situ*, thereby changing the otherwise lethal disease to a chronic ailment, and eliminating the neurologic damage of more conventional therapies. The invasive nature of glial tumors, as well as the neurological sequelae of extensive resection, make this type of approach appealing. Further studies, intended to elucidate the transcriptional regulation of CD81 in astrocytes, will provide insights into potential pharmacotherapeutics.

[0226] The data presented here clearly show the importance of CD81 in normal, neuron-induced astrocyte proliferative regulation. This observation reveals, to some extent, the mechanism(s) underlying the way in which the ratio of neurons and astrocytes is established and maintained in the adult CNS. Further delineation of the molecular mechanisms that control the dynamic interactions between these cell types is critical to the development of a more complete understanding of the means by which the mature nervous system achieves and maintains numerical homeostasis, and the way in which this balance may be restored when the nervous system moves out of equilibrium.

[0227] The inventor also has shown herein that astrocytes express a receptor, CD81, which is absolutely required for neuron-induced growth arrest. The CD81 ligand has been shown herein to be a novel transmembrane tyrosine kinase, NrS1, that is itself rapidly phosphorylated in response to CD81 signaling. Following this rapid phosphorylation, a transmembrane form of Nrg is cleaved (*i.e.*, the extracellular domain of Nrg, which binds erbB2 and erbB4 on the astrocyte), resulting in elevations of intracellular cAMP levels and astrocyte maturation. Accordingly, there is forward signaling to the astrocyte. The binding of the astrocyte/CD81 mimetic, GM1109, to the neuronal surface drives cleavage of Nrg and the translocation of the ICD_{Nrg} to the neuronal nucleus, thereby promoting neuronal survival. Accordingly, there is also reverse signaling to the neuron. This bi-directional signaling is depicted in FIG. 19.

[0228] Taken together, the inventor's observations suggest three distinct therapeutic possibilities: (1) use of the CD81 mimetic, GM1109, or derivatives thereof, in the direct treatment of neural degeneration, including neurodegenerative disease; (2) use of agonists of NrS1 signaling in the treatment of conditions associated with a defect in astrocyte proliferation, including astrocytosis; (3) and re-expression of CD81 in astrocytoma cells in order to re-establish growth control.

Example 2. Characterization of NrS1.

Introduction

[0229] Unlike the vast majority of cells in the body, neurons are incapable of dividing or being replaced over a lifetime, suggesting unique mechanism(s) which provide continuous support and cellular repair. In contrast, astrocytes, the cells which provide much of the trophic support and provide the physical scaffolding in which the neurons reside, are able to re-enter the cell cycle at virtually any point in their history, and do so in response to trauma and disease (15, 28, 49). However, despite this proliferative ability, the number of astrocytes remains largely unchanged throughout life (23, 24, 25), thereby posing a conundrum as to the mechanisms(s) that regulate of their growth.

[0230] The significance of how this mitotic quiescence is established and maintained is enormous, as there are major sequelae, both positive and negative, that result from astrocyte proliferation in the adult. These include gliotic responses to injury, the secondary block of axonal migration and neuronal remodeling, collateral damage to otherwise intact tissue from astrocytotic spillover, and the possibility of spontaneous mutations and/or viral integration in astrocytes as

they transit the cell cycle, all of which can lead to astrocytoma. We have recently described results in which we have discovered the identity of the astrocyte receptor required for neuronal signal, termed CD81. We then turned our attention to the identification of the neuronal ligand for CD81, which we have termed NrS1. We have utilized three different approaches to identifying this protein, including protein pull-down followed by mass spectrometry analysis, expression cloning and cDNA cloning. The results from these studies all converged on the identification of a gene previously identified as EHK1, a member of the eph class of receptor tyrosine kinases.

[0231] Eph-related receptors are membrane-bound protein tyrosine kinases that interact with membrane-bound ligands on adjacent cells. Thus, Ephs transduce signals in a manner that is dependent on cell-to-cell contact, and which allows for forward and reverse signaling between the two cell-types after binding. Ehk1 was identified in 1993 (46), and since its identification, only two additional papers have appeared in the literature. One of these, by Taylor and colleagues (48), demonstrated expression of Ehk1 to be overlapping and virtually identical to the expression pattern we demonstrate for NrS1. Importantly, these authors argue that Ehk1/NrS1 expression increases upon neural cell-cell contact, and mediates the aggregation of these cells. This is the basis by which we identified the NrS1/Ehk-1 binding partner, CD81 (34, 45). The only other published manuscript describes the extensive splice variations of Ehk1 expressed in the nervous system (47), which we also found, and have expand upon, as described below. Finally, and perhaps most strikingly, the only cell-type other than CNS neurons that have been shown to inhibit astrocyte proliferation are glioma cells (28), which also express Ehk1 (48). Taken together, these data support to identification of Ehk1 as NrS1. Below we describe the isolation of Ehk-1 from human brain and from a human fetal brain cDNA library.

Methods and Results

Approaches to identification of NrS1

[0232] Protein-protein interactions. We have taken advantage of the specific interaction between NrS1 and GM1109 to pull down NrS1-GM1109 complexes from a whole brain membrane protein preparation. In brief, solubilized membrane preparations from human fetal brain were incubated with GM1109, the complexes were purified by binding to glutathione/agarose beads, and eluted with free glutathione. The complexes were analyzed by two dimensional gel electrophoresis (isoelectric focusing in the first dimension and size fractionation by SDS-PAGE in the second dimension) (FIG. 26). In this analysis a total of 4 spots were

consistently obtained which were isolated and analyzed by mass-spectrometry. All the bands had ions that identified Ehk-1. The skilled artisan would understand that apparent molecular mass observed in these experiments could be the result of degradation or expression of splice variants.

[0233] Expression cloning. Parallel experiments screened a human fetal brain lambda phage cDNA expression library with GM1109. A total of 16 clones were isolated by positive binding to GM1109. The identity of the clones was confirmed after excision reactions and sequencing of the cDNAs.

[0234] cDNA cloning by homology. To avoid the avoid the possibility of false positives that could have arisen by expression screening with the bait, we took advantage of the fact that NrS1 is a protein tyrosine kinase and developed an strategy to identify specifically protein tyrosine kinases among the positive clones. We used degenerate oligonucleotide primers corresponding to the amino acid motifs IHRDL and DVWSFG conserved among many tyrosine kinases in regions VI and IX, respectively, of the catalytic domain (50). These primers were used in polymerase chain reactions (PCR) performed on DNA templates from the 16 positive clones that resulted from the protein library screening. Eight clones generated the expected 200bp PCR fragment upon amplification and they were sequenced to identify the respective tyrosine kinase proteins. A novel exon 5' to the published sequence of EHK1 was also identified by these methods (FIG. 29).

[0235] The three independent approaches lead to the identification of the receptor tyrosine kinase, Ehk1 as NrS1.

[0236] Distribution of NrS1/Ehk1 and protection of retinal ganglion cells and axons in a retinal stroke model. Histological studies established that NrS1/Ehk1 is widely distributed in cortical, subcortical, retinal, and enteric neurons. (FIGS. 30-32). The presence of NrS1 in retina allowed the use of an occular stroke model to determine the ability of the active CD81 fragment GM1416 to protect against stroke. Using this model, the inventor determined that GM1416 did protect retinal ganglion when stroke was induced (FIG. 36). Additionally, axons in the inner plexiform layer (IPL) of the retina were protected in the retinal stroke model. This further indicates that CD81 or its active fragments are useful in the treatment of stroke.

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[0288] Other embodiments within the scope of the claims herein will be apparent to one skilled in the art from consideration of the specification or practice of the invention as disclosed herein. It is intended that the specification be considered exemplary only, with the scope and spirit of the invention being indicated by the following claims.

[0289] In view of the above, it will be seen that the several advantages of the invention are achieved and other advantages attained.

[0290] As various changes could be made in the above methods and compositions without departing from the scope of the invention, it is intended that all matter contained in the above description and shown in the accompanying drawings shall be interpreted as illustrative and not in a limiting sense.

[0291] All references cited in this specification are hereby incorporated by reference. The discussion of the references herein is intended merely to summarize the assertions made by the authors and no admission is made that any reference constitutes prior art. Applicants reserve the right to challenge the accuracy and pertinence of the cited references.

What is claimed is:

1. A method for enhancing survival of neurons, comprising contacting the neurons with CD81 protein, a CD81 derivative or stabilized variants thereof, in an amount effective to enhance survival of the neurons.

2. The method of claim 1, wherein the neurons are contacted with a CD81 derivative, the CD81 derivative comprising the amino acid sequence of FIG. 38E.

3. The method of claim 2, wherein the CD81 derivative comprises GM1109, GM1416 or a stabilized variant thereof.

4. The method of claim 1, wherein the contacting is effected *in vitro*.

5. The method of claim 1, wherein the contacting is effected *in vivo* in a mammal.

6. The method of claim 5, wherein the contacting is effected *in vivo* in the mammal by administering to the mammal the CD81 protein, the CD81 derivative or stabilized variants thereof.

7. The method of claim 6, wherein the CD81 protein or CD81 derivative is administered to the mammal by oral administration, parenteral administration, sublingual administration, transdermal administration, or osmotic pump.

8. The method of claim 5, wherein the CD81 or CD81 derivative is administered to the mammal by administering a nucleic acid encoding the CD81 or CD81 derivative, in a manner permitting expression of the CD81, CD81 derivative or stabilized variants thereof.

9. The method of claim 5, wherein the mammal is a human.

10. The method of any one of claims 5-9, wherein the mammal has neural degeneration.

11. The method of claim 10, wherein the neural degeneration is a primary neurologic condition or an acquired secondary effect of a non-neural dysfunction.

12. The method of claim 11, wherein the neural degeneration is a primary neurologic condition selected from the group consisting of Alzheimer's disease, amyotrophic lateral sclerosis, Binswanger's disease, Huntington's chorea, multiple sclerosis, myasthenia gravis, Parkinson's disease, and Pick's disease.

13. The method of claim 11, wherein the neural degeneration is an acquired secondary effect of a non-neural dysfunction selected from the group consisting of cerebral palsy, congenital hydrocephalus, muscular dystrophy, stroke, and vascular dementia.

14. The method of claim 10, wherein the neural degeneration is associated with a demyelinating condition.

15. The method of claim 14, wherein the demyelinating condition is selected from the group consisting of acute disseminated encephalomyelitis (ADEM), acute transverse myelitis, acute viral encephalitis, adrenoleukodystrophy (ALD), adrenomyeloneuropathy, AIDS-vacuolar myelopathy, HTLV-associated myelopathy, Leber's hereditary optic atrophy, multiple sclerosis, progressive multifocal leukoencephalopathy (PML), subacute sclerosing panencephalitis, and tropical spastic paraparesis.

16. A method for treating neural degeneration in a mammal in need of treatment, comprising activating NrS1 in the mammal.

17. The method of claim 16, wherein NrS1 is activated in the mammal by administering to the mammal CD81 protein, a CD81 derivative or a stabilized variant thereof.

18. The method of claim 17, wherein the CD81 protein, CD81 derivative or stabilized variant thereof is administered to the mammal in an amount effective to treat the neural degeneration in the subject.

19. The method of claim 17, wherein the mammal is administered a CD81 derivative, the CD81 derivative comprising the amino acid sequence of FIG. 38E.

20. The method of claim 19, wherein the CD81 derivative comprises GM1109, GM1416 or a stabilized variant thereof.

21. The method of claim 17, wherein the CD81 protein or CD81 derivative is administered to the subject by oral administration, parenteral administration, sublingual administration, transdermal administration, or osmotic pump.

22. The method of claim 16, wherein the CD81 or CD81 derivative is administered to the mammal by administering a nucleic acid encoding the CD81 or CD81 derivative, in a manner permitting expression of the CD81, CD81 derivative, or stabilized variant thereof.

23. The method of claim 16, wherein the neural degeneration is a primary neurologic condition or an acquired secondary effect of a non-neural dysfunction.

24. The method of claim 23, wherein the neural degeneration is a primary neurologic condition selected from the group consisting of Alzheimer's disease, amyotrophic lateral sclerosis (Lou Gehrig's Disease), Binswanger's disease, Huntington's chorea, multiple sclerosis, myasthenia gravis, Parkinson's disease, and Pick's disease.

25. The method of claim 23, wherein the neural degeneration is an acquired secondary effect of a non-neural dysfunction selected from the group consisting of cerebral palsy, congenital hydrocephalus, muscular dystrophy, stroke, and vascular dementia.

26. The method of claim 16, wherein the neural degeneration is associated with a demyelinating condition.

27. The method of claim 26, wherein the demyelinating condition is selected from the group consisting of acute disseminated encephalomyelitis (ADEM), acute transverse myelitis, acute viral encephalitis, adrenoleukodystrophy (ALD), adrenomyeloneuropathy, AIDS-vacuolar myelopathy, HTLV-associated myelopathy, Leber's hereditary optic atrophy, multiple sclerosis, progressive multifocal leukoencephalopathy (PML), subacute sclerosing panencephalitis, and tropical spastic paraparesis.

28. A method for inhibiting proliferation of astrocytes, comprising contacting the astrocytes with an amount of NrS1 protein or NrS1 derivative effective to inhibit proliferation of the astrocytes.

29. The method of claim 28, wherein the NrS1 protein or NrS1 derivative comprises the amino acid sequence of FIG. 28, or a biologically active fragment thereof.

30. The method of claim 28, wherein the contacting is effected *in vitro*.

31. The method of claim 28, wherein the contacting is effected *in vivo* in a mammal.

32. The method of claim 31, wherein the contacting is effected *in vivo* in the mammal by administering an NrS1 protein an NrS1 derivative or a stabilized variant thereof to the mammal.

33. The method of claim 32, wherein the NrS1 protein or NrS1 derivative is administered to the mammal by oral administration, parenteral administration, sublingual administration, transdermal administration, or osmotic pump.

34. The method of claim 31, wherein the contacting is effected by administering a nucleic acid encoding the NrS1 or NrS1 derivative, in a manner permitting expression of the NrS1.

35. The method of claim 31, wherein the mammal is a human.

36. The method of claim 35, wherein the human has a condition associated with a defect in astrocyte proliferation.

37. The method of claim 36, wherein the condition associated with a defect in astrocyte proliferation is astrocytosis.

38. A method for treating a condition associated with a defect in astrocyte proliferation in a subject in need of treatment, the method comprising activating CD81 in the subject.

39. The method of claim 38, wherein CD81 is activated in the subject by administering to the subject NrS1 protein, a NrS1 derivative, or a stabilized variant thereof.

40. The method of claim 39, wherein the NrS1 protein, NrS1 derivative or stabilized variant thereof is administered to the subject in an amount effective to treat the condition associated with a defect in astrocyte proliferation in the subject.

41. The method of claim 38, wherein the condition associated with a defect in astrocyte proliferation is astrocytosis.

42. The method of claim 39, wherein the NrS1 protein or NrS1 derivative is administered to the subject by oral administration, parenteral administration, sublingual administration, transdermal administration, or osmotic pump.

43. A method for determining whether a mammal has an astrocytoma, comprising assaying for CD81 expression in a diagnostic sample of cells of astrocytic lineage of the mammal, wherein no detection of expression of CD81 in cells of astrocytic lineage of the subject is diagnostic of an astrocytoma.

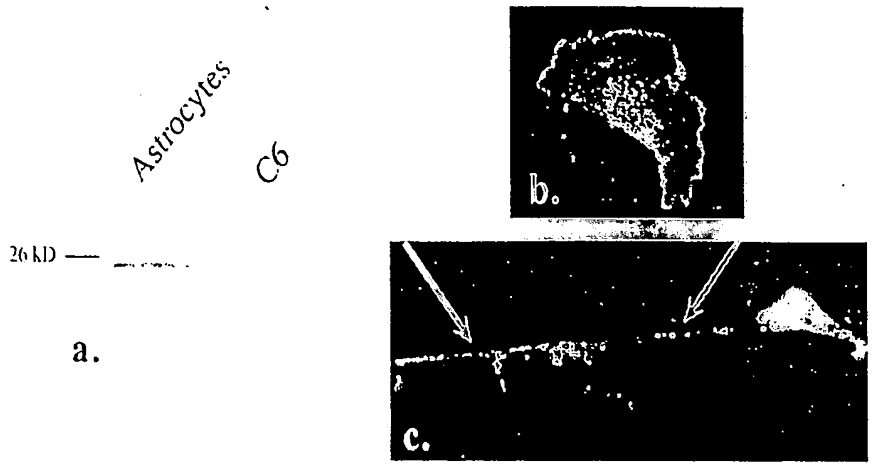
44. The method of claim 43, wherein the mammal is a human.

45. An isolated nucleic acid of less than 10,000 nucleotides, comprising the nucleotide sequence of FIG. 29.

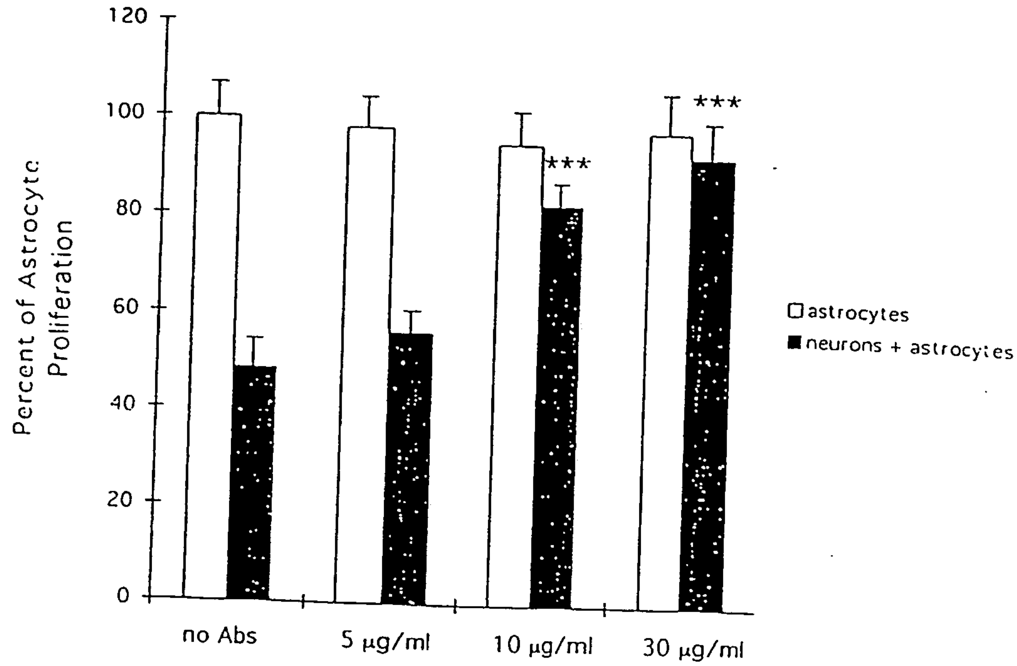
46. The isolated nucleic acid of claim 45, wherein the nucleic acid is less than 5000 nucleotides.

47. The isolated nucleic acid of claim 45, wherein the nucleic acid is less than 1000 nucleotides.

48. The isolated nucleic acid of claim 45, wherein the nucleic acid is less than 200 nucleotides.

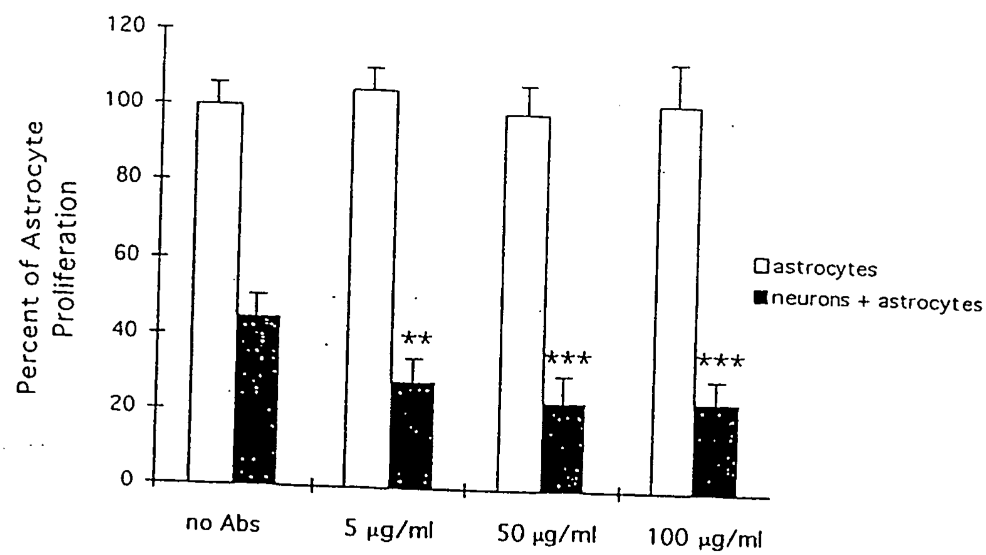


Figs. 1a, b, and c



a.

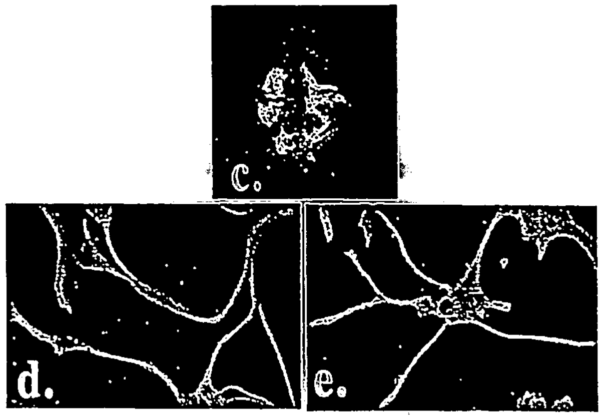
mAb Eat1



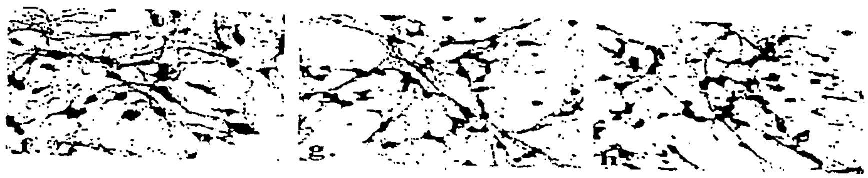
b.

mAb 2F7

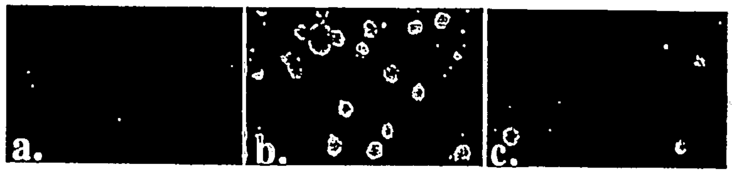
Figs. 2a and b



Figs. 2c, d, and e



Figs. 2f, g, and h



Figs. 3a, b, and c

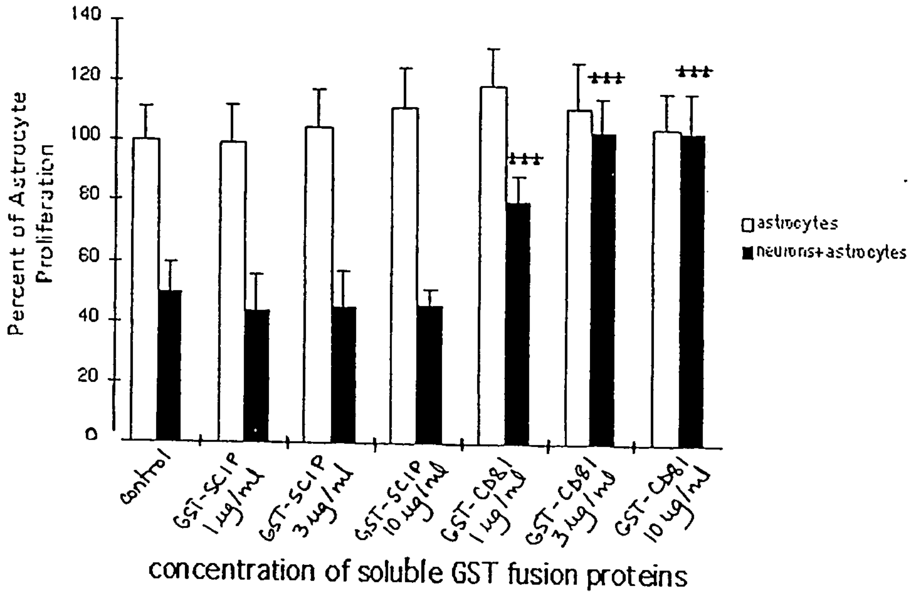
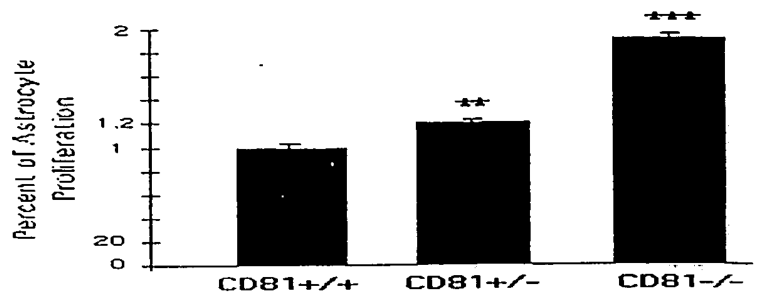

Percent of Astrocyte Proliferation
140
120
100
80
60
40
20
0
astrocytes
neurons+astrocytes
control
GST-SCIP 1ug/ml
GST-SCIP 3ug/ml
GST-SCIP 10ug/ml
GST-CD81 1ug/ml
GST-CD81 3ug/ml
GST-CD81 10ug/ml
concentration of soluble GST fusion proteins

Fig. 4


Percent of Astrocyte Proliferation
2
1.2
1
20
0
CD81+/+
CD81+/-
CD81-/-

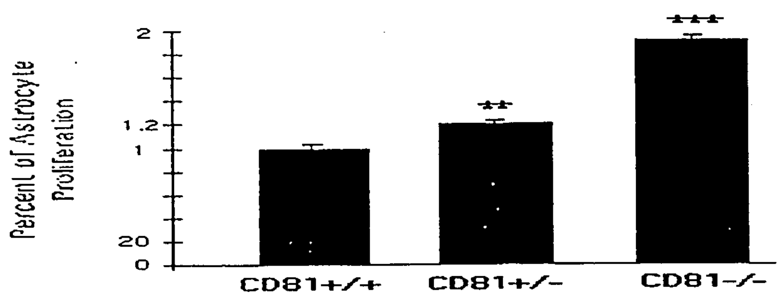

Percent of Astrocyte Proliferation
2
1.2
1
20
0
CD81+/+
CD81+/-
CD81-/-

Fig. 5

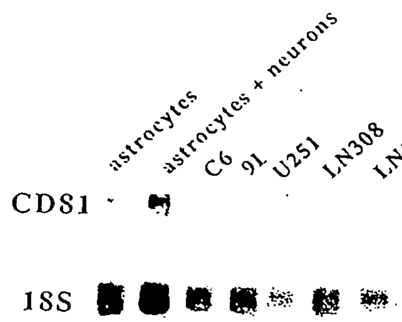

astrocytes
astrocytes + neurons
C6
9L
U251
LN308
LN18
CD81
18S

Fig. 6

aaatacctgctcttcgtcttcaatttcgtcttctggctggctggaggcgtgatcctaggtgtagctctgtggttgcgtca
tgatccacagaccaccagcctgctgtacctggaactgggaaacaaaccggcacccaacaccttctacgtgggcatctaca
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tggtgctgtgctgtggcatccggaacagctccgtgtactgag

Fig. 7

KYLLFVFNFVFWLAGGVILGVALWLRHDPQTTSLLYLELGNKPAPNTFYVGIYILIAVGAVM
MFVGFLGCYGAIQESQCL
LGTFFTCLVILFACEVAAGIWGFVNKDQIAKDVKQFYDQALQQAVMDDDANNAKAVVKTF
HETLNCCGSNALTTLTTTIL
RNSLCPSGGNILTPLLQQDCHQKIDELFSGKLYLIGIAAIVVAVIMIFEMILSMVLCCGIRNSS
VY

Fig. 8

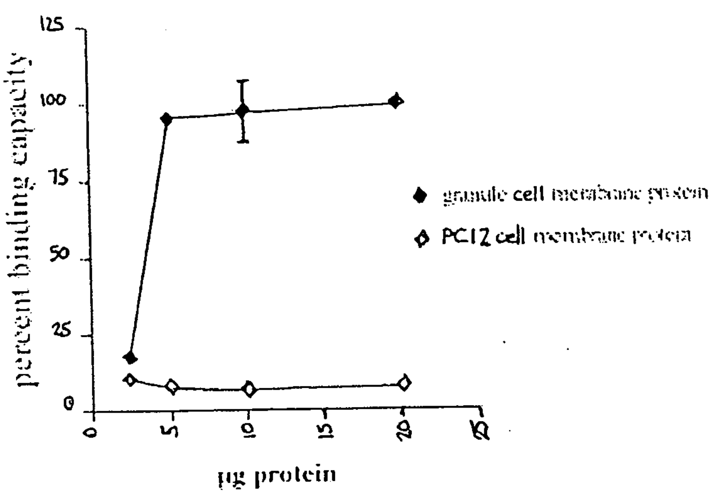
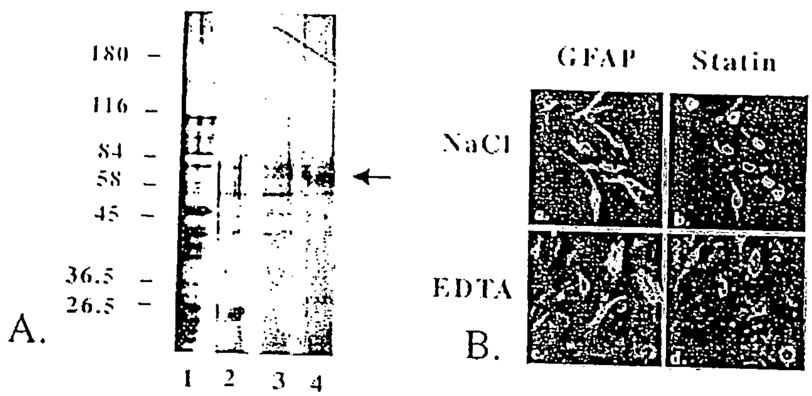

125
100
75
50
25
0
percent binding capacity
0
5
10
15
20
25
µg protein
granule cell membrane protein
PC12 cell membrane protein

Figure 9



Figures 10a and 10b

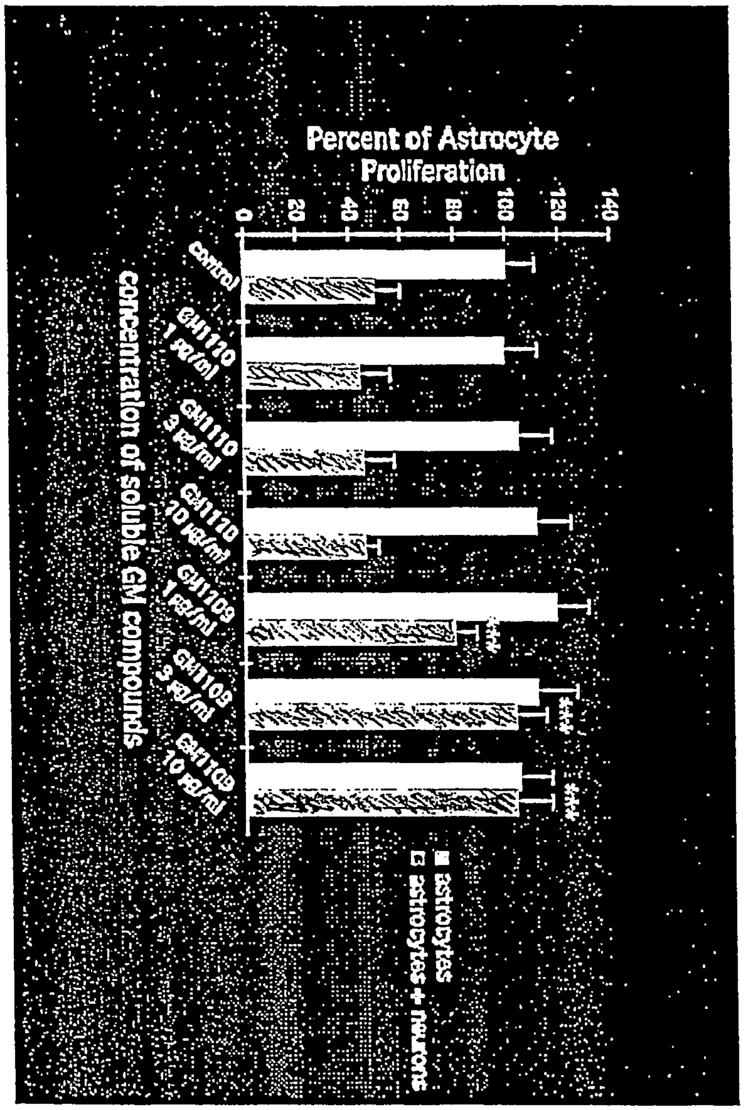

Percent of Astrocyte Proliferation
0
20
40
60
80
100
120
140
control
GH1110 1 µg/ml
GH1110 3 µg/ml
GH1110 10 µg/ml
GH1109 1 µg/ml
GH1109 3 µg/ml
GH1109 10 µg/ml
astrocytes
astrocytes + neurons
concentration of soluble GM compounds

Figure 11

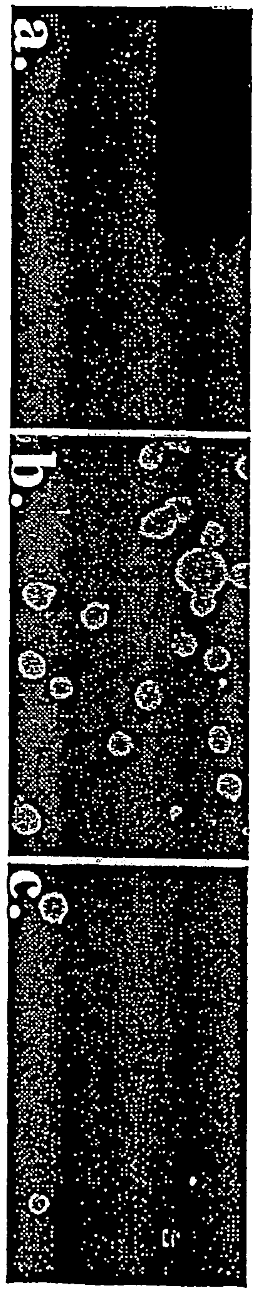
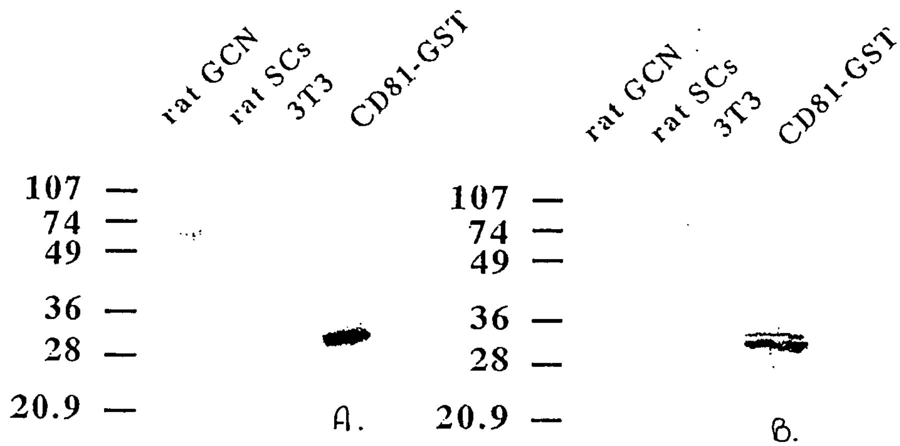


Figure 12



Figures 13a and 13b

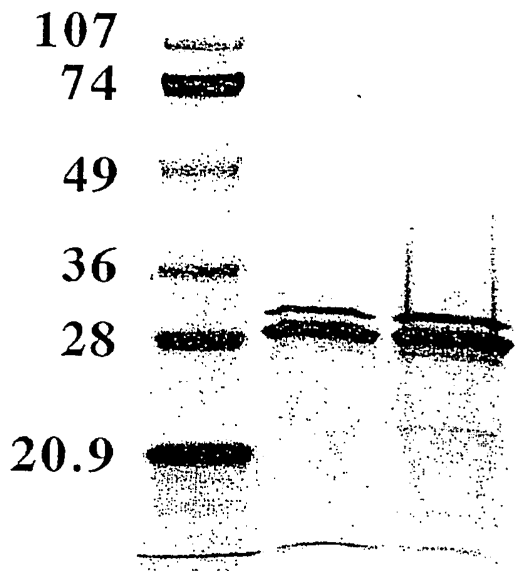


Figure 14

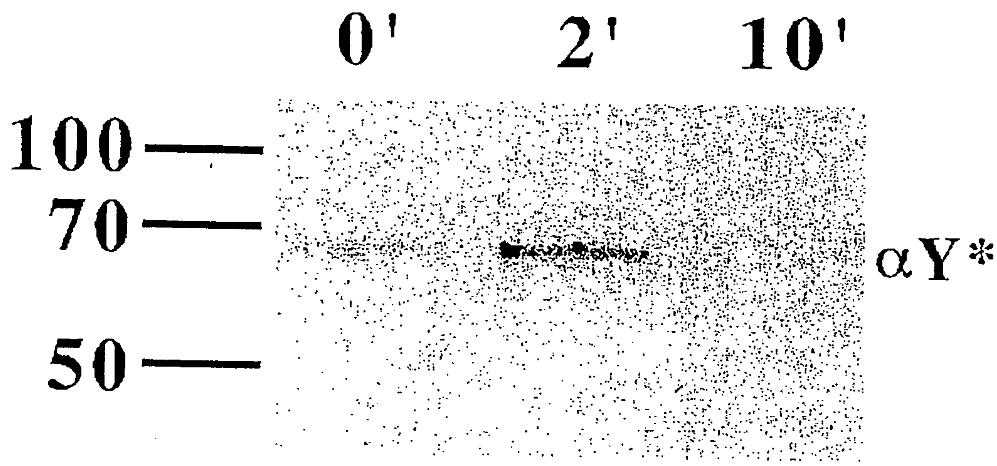
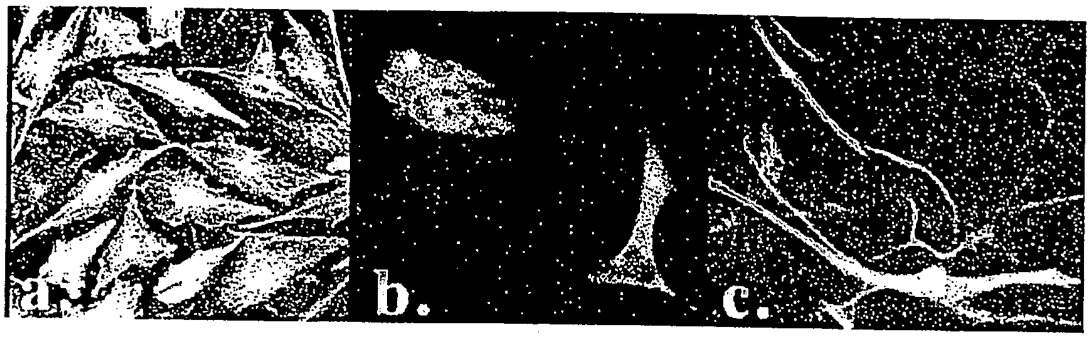
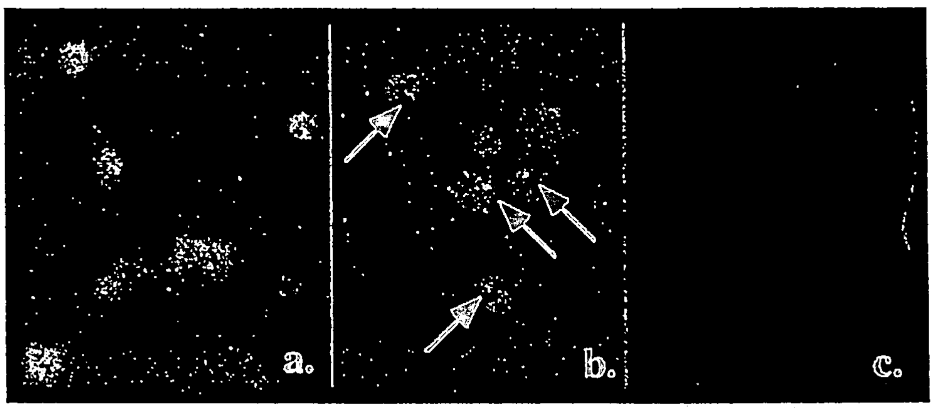


Figure 15



Figures 16a, b, and c



Figures 17a, b, and c

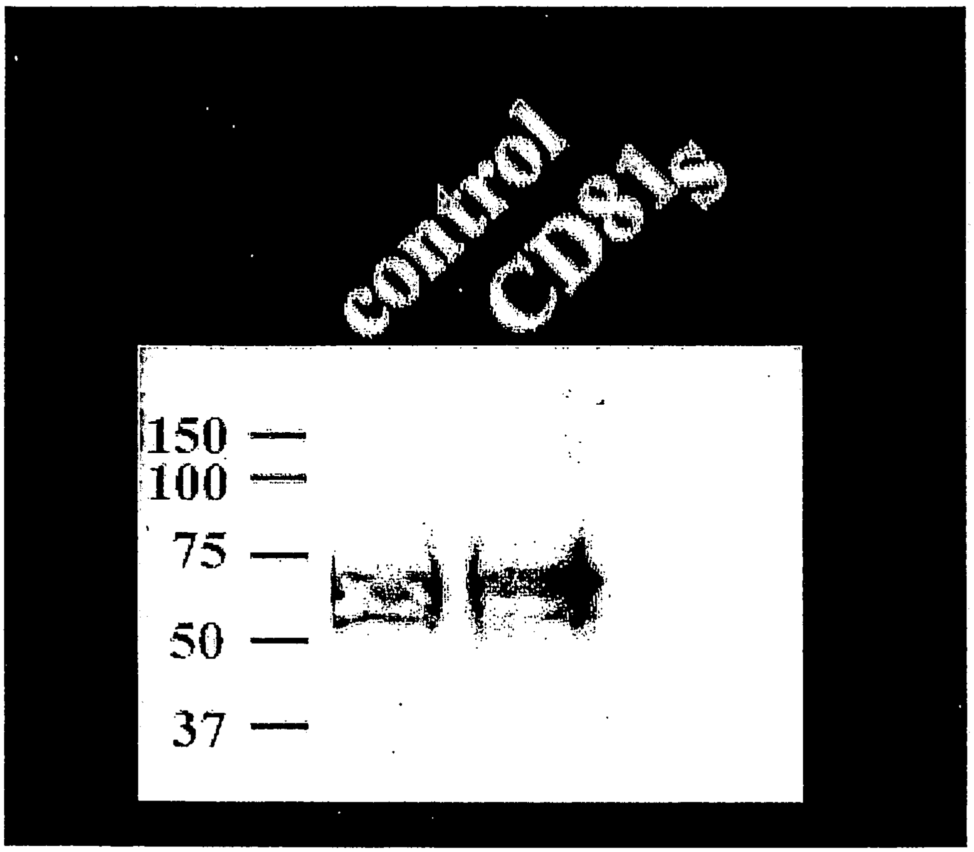


Figure 17d

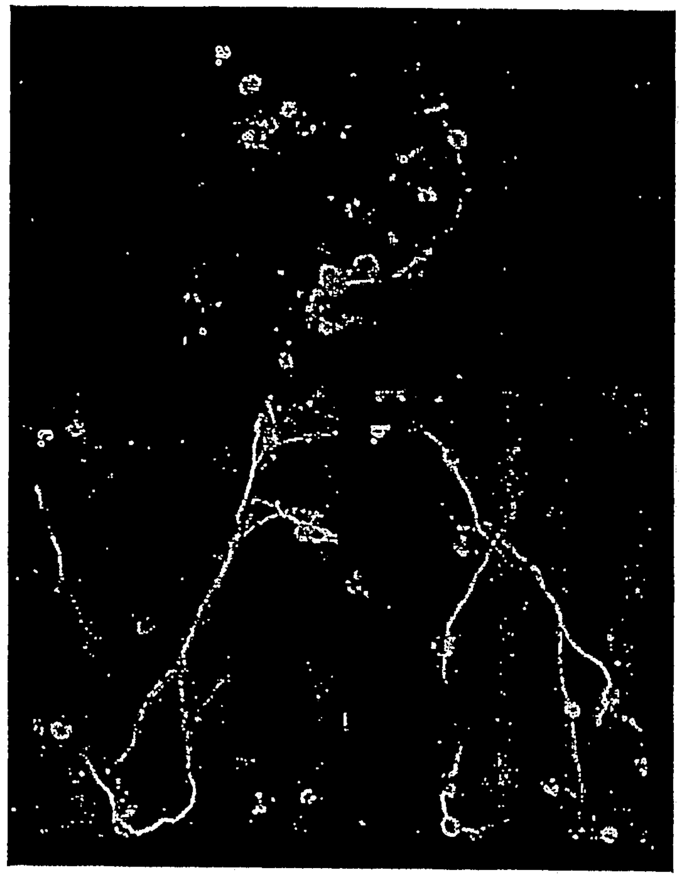
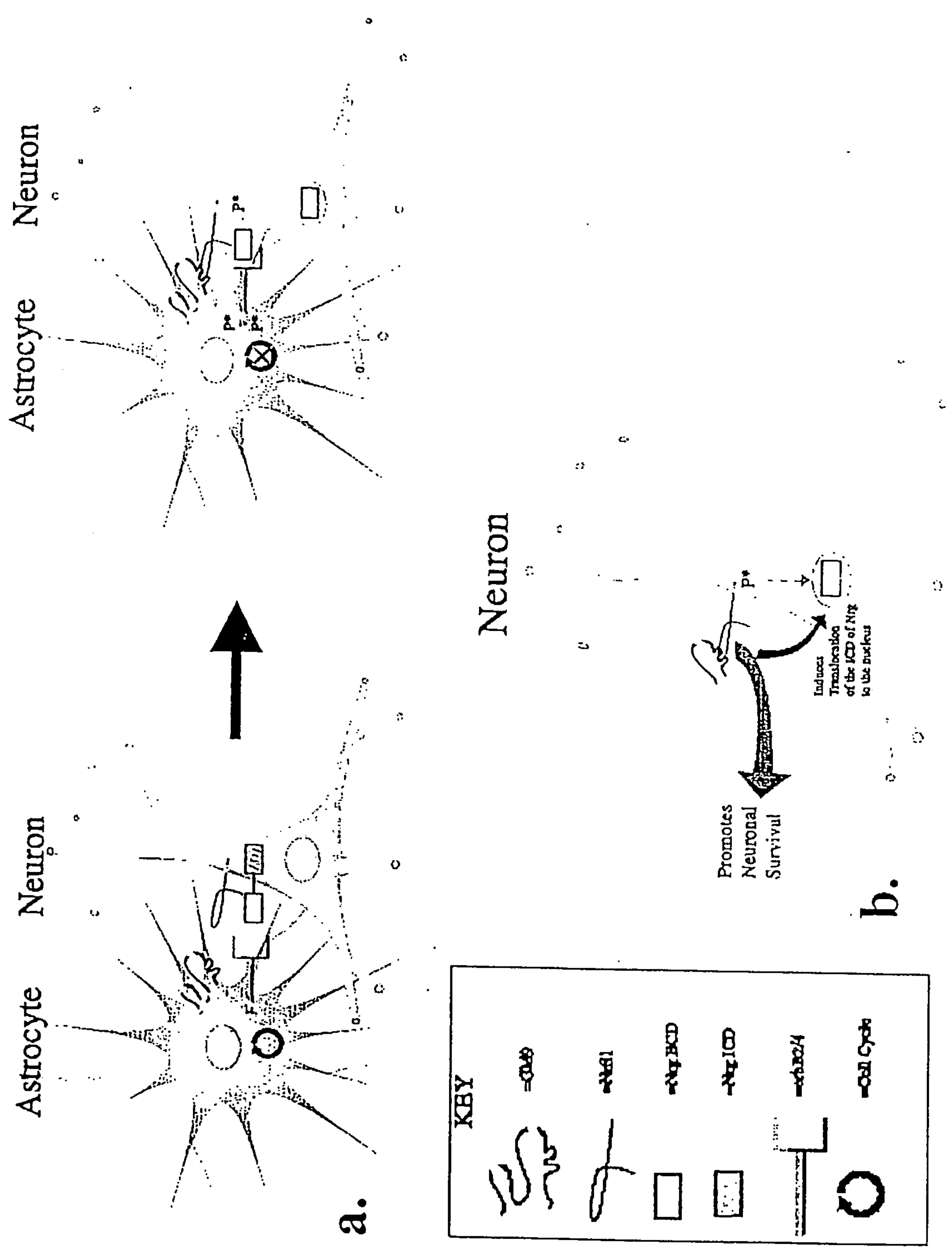


Figure 18



Figures 19a and 19b

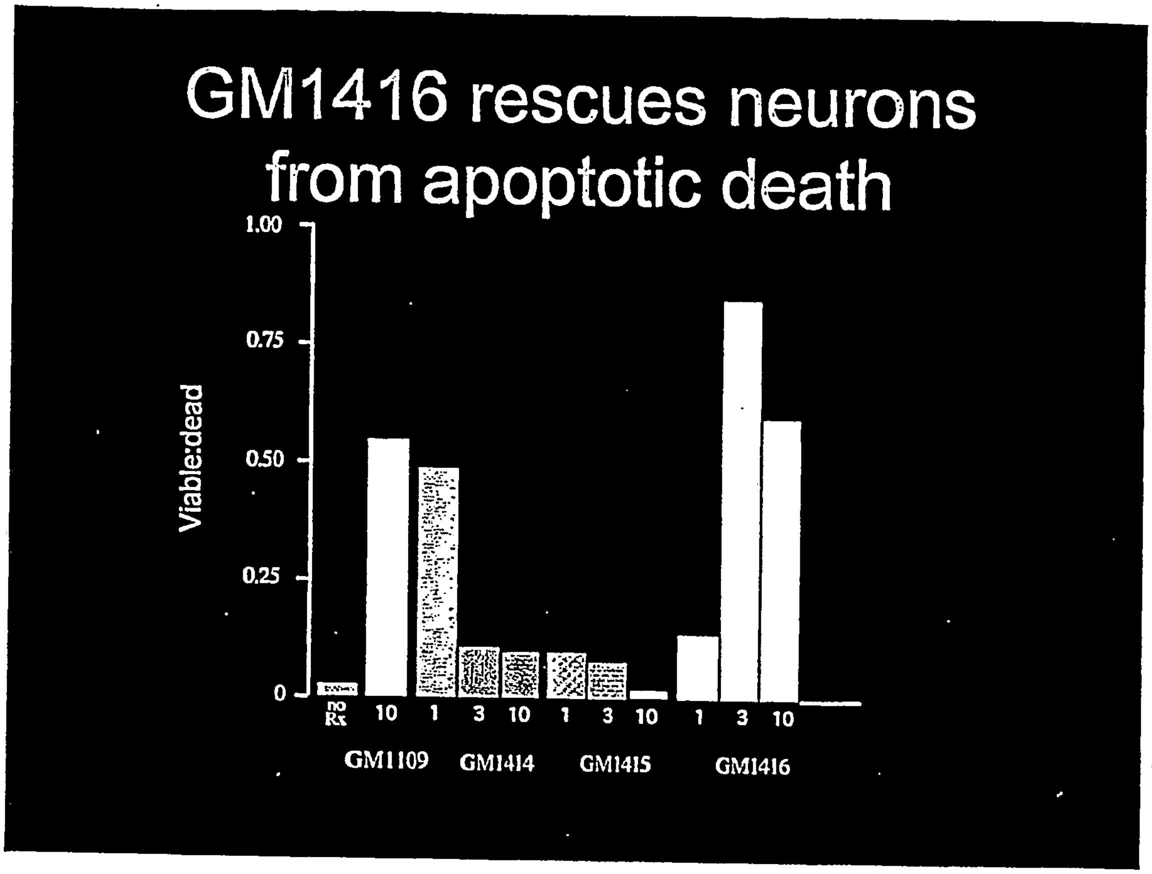

GM1416 rescues neurons from apoptotic death
Viable:dead
1.00
0.75
0.50
0.25
0
no Rx
10
1
3
10
1
3
10
1
3
10
GM1109
GM1414
GM1415
GM1416

Figure 20a

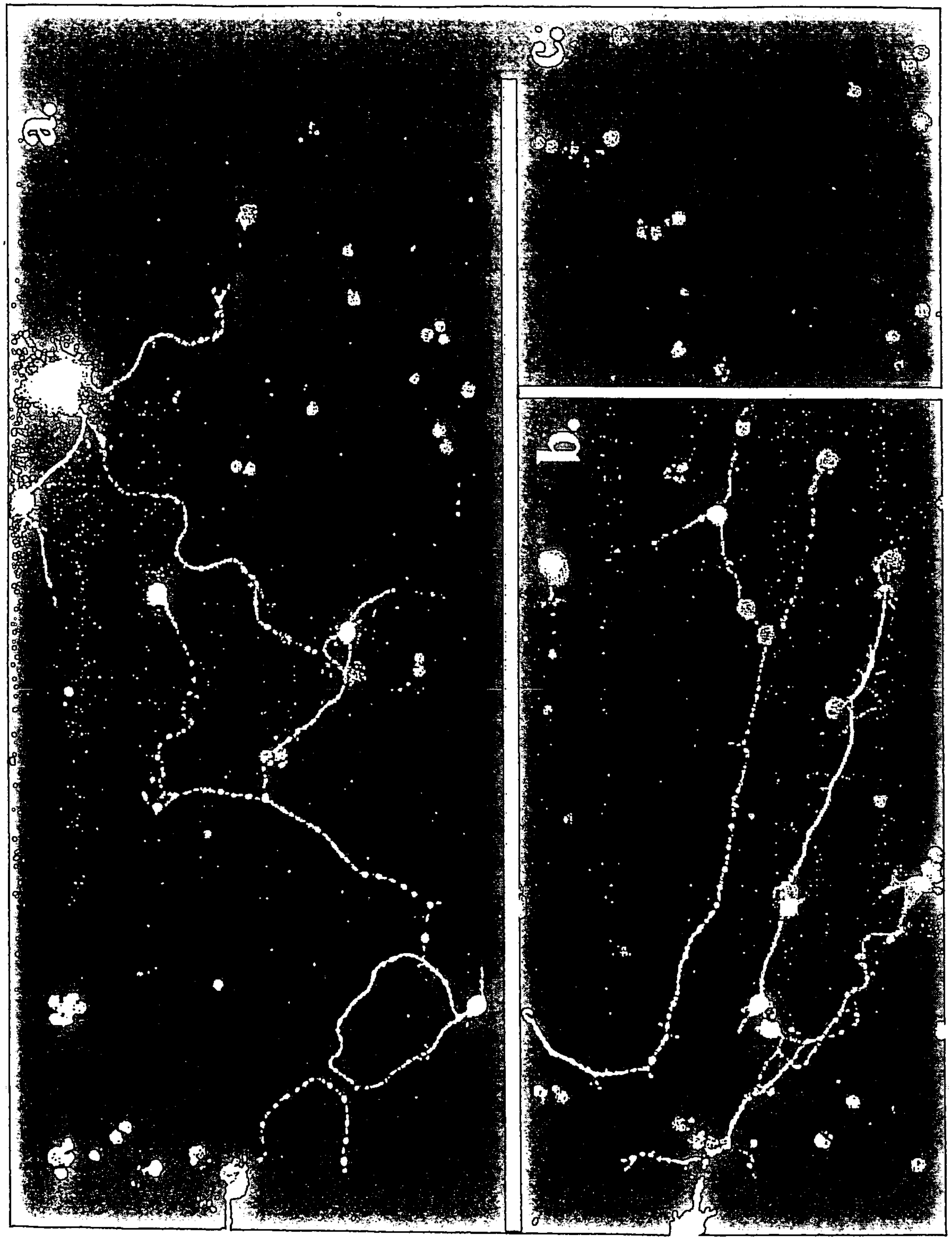
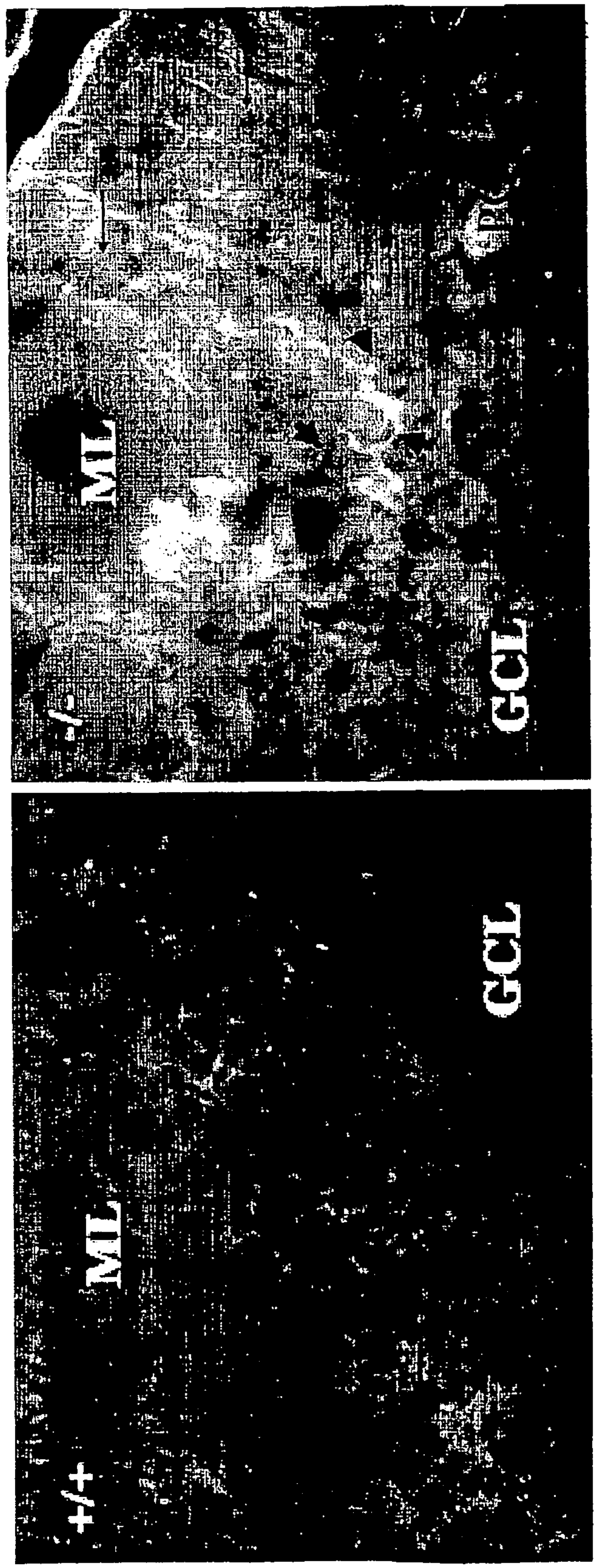


Figure 20b

FIG.21



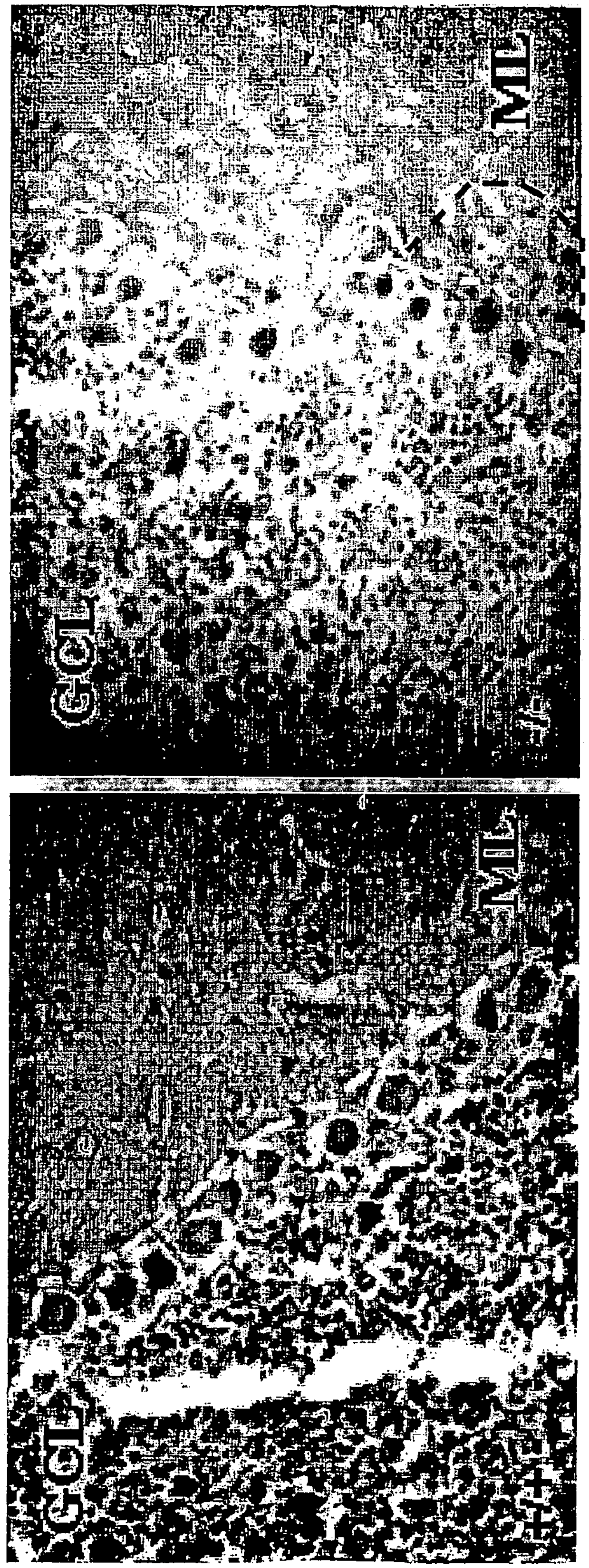


FIG. 22

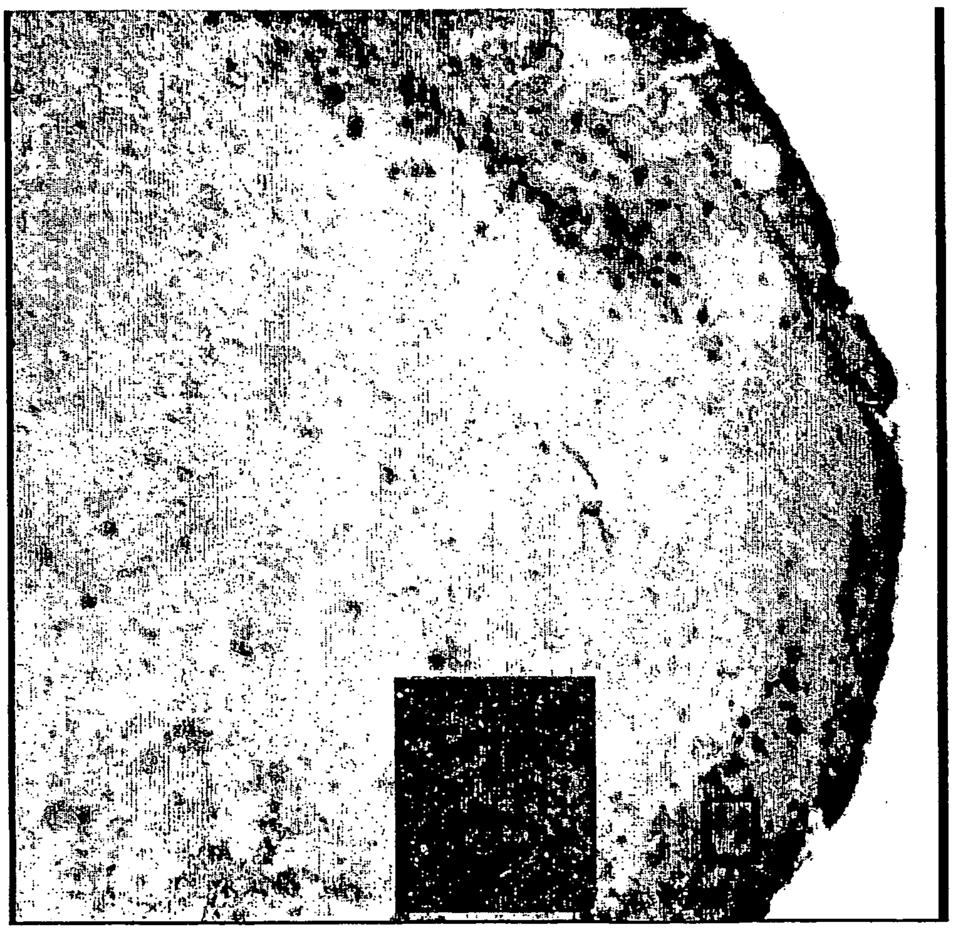
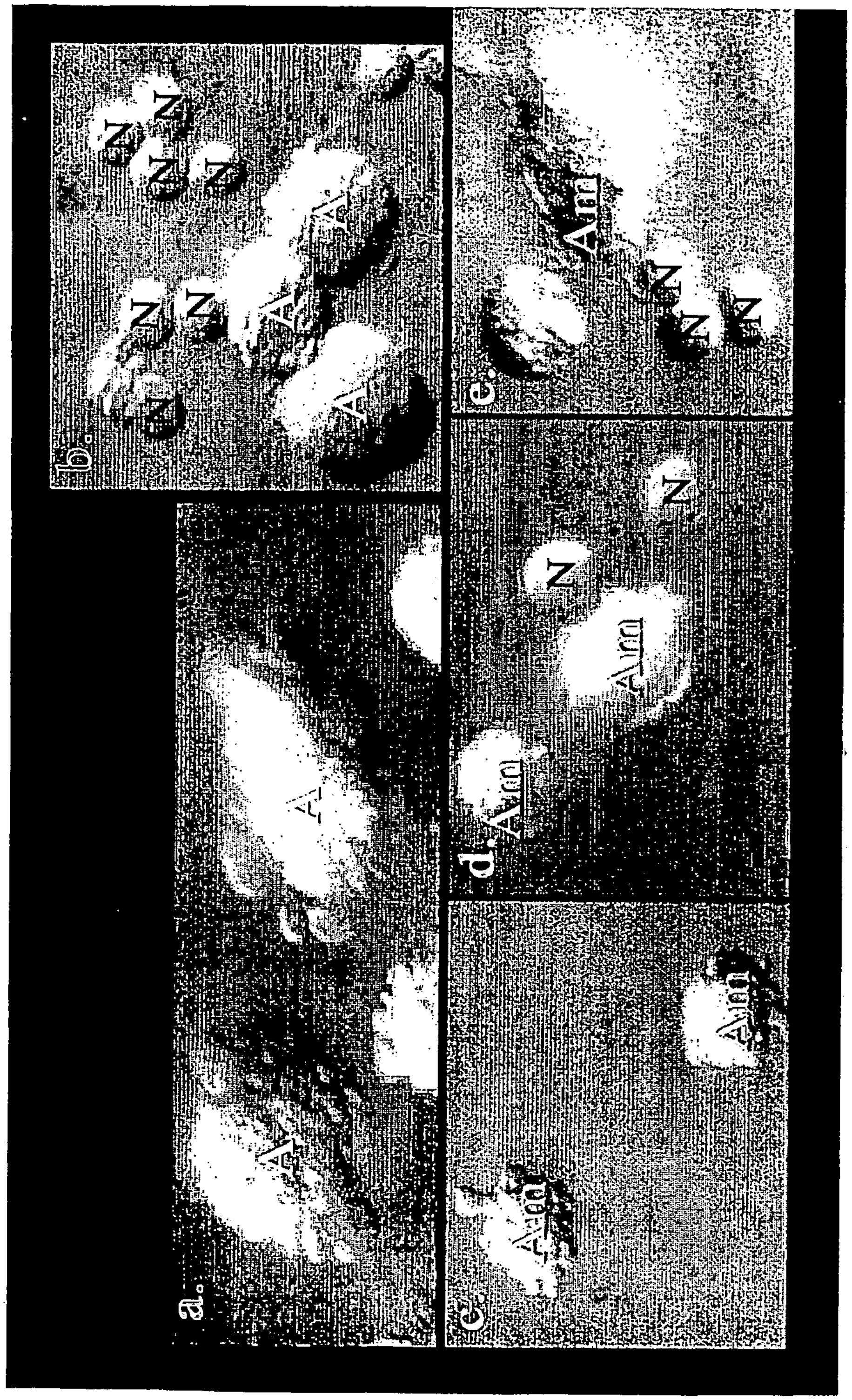


FIG. 23

FIG. 24



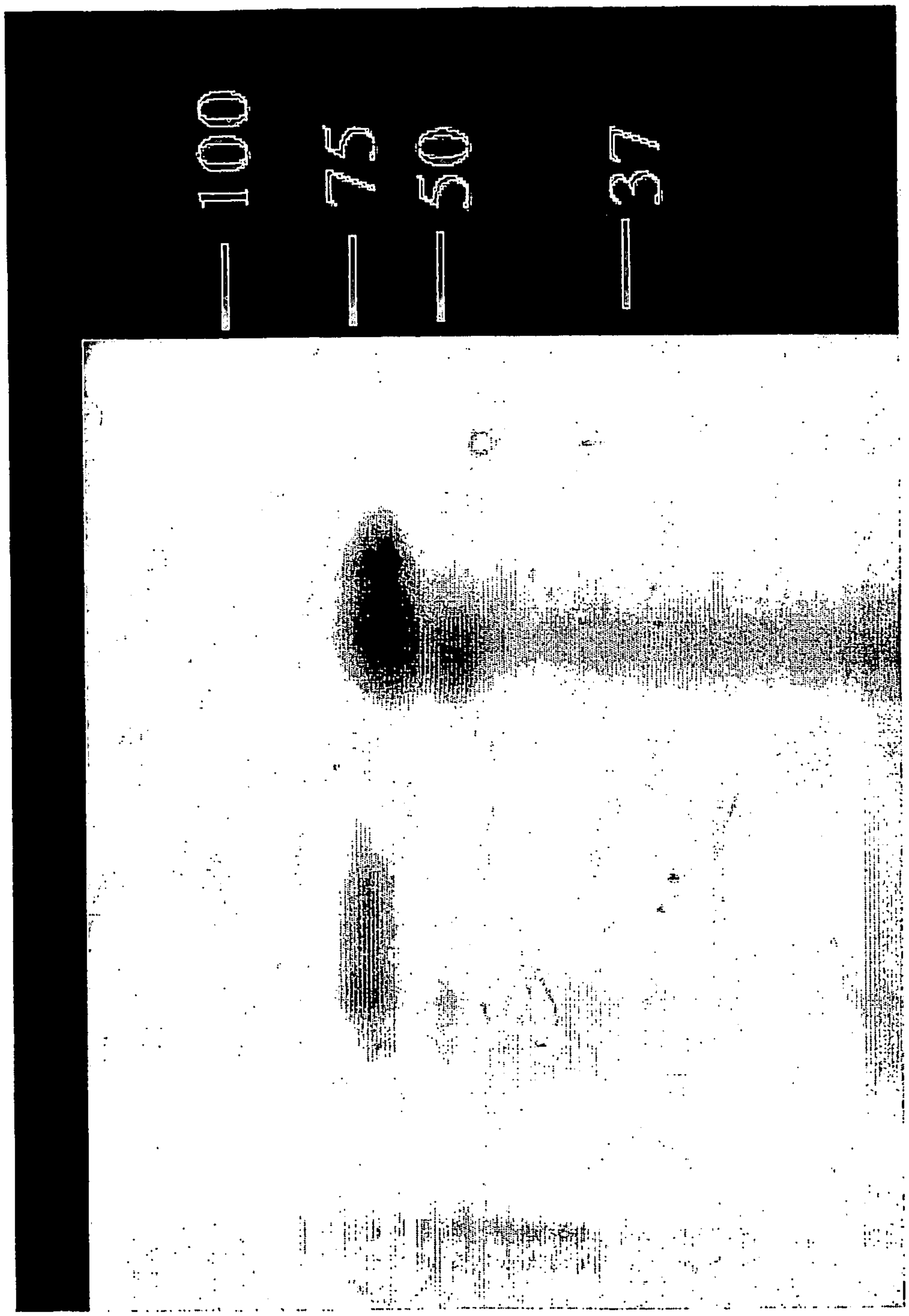


FIG. 26

FIG. 27 - The published nucleotide sequence of EHK1.

```
   1 gtccctcccc tcttcagcac tcagcccgca gctatttcct tctgccagtc tctttgaact
  61 ctggatcttt gcttttgctc gctgctctcc tgtttttcat tctccacatt ttctcaagtc
 121 ctctttcttt atccttagcc accctgcttt tttcctcctt ttttaaaaaa tcggagattt
 181 cgtcttaaaa tgatttgcct tccttacctt cgtccatttc aacactgaag gctgcaaaga
 241 accttcacct ttcccctagt ggtatttaaa aattctcaat ccgtaaaaag tctttttgaa
 301 aggcaaagga acaggaccca ggaccctctc gacacccttg atccgagtcc agatctgcac
 361 tagcaaccag aactaatatt tcatttaacc ccaccaaagg gggaggcgag aggagccaga
 421 agcaaacttc agctgtctca gccggatccg tggttcctac atttggagga gccgcgtgcc
 481 agaaggcgta ggaccccaag gggggacaag gaggactccc gagtctccct tcgccgctct
 541 gcgaggccga agcggtggac tgagccgctc gggacagcgg caccggagga ggctcggaga
 601 agatgcgggg ctcggggccc cggggtgcgg gacgccggcg gcccccaagc ggcggcggcg
 661 acaccccccat caccccagcg tccctggccg gctgctactc tgcacctcga cgggctcccc
 721 tctggacgtg ccttctcctg tgcgccgcac tccggaccct cctggccagc cccagcaacg
 781 aagtgaattt attggattca cgcactgtca tgggggacct gggatggatt gcttttccaa
 841 aaaatgggtg ggaagagatt ggtgaagtgg atgaaaatta tgcccctatc cacacatacc
 901 aagtatgcaa agtgatggaa cagaatcaga ataactggct tttgaccagt tggatctcca
 961 atgaaggtgc ttccagaatc ttcatagaac tcaaatttac cctgcgggac tgcaacagcc
1021 ttcctggagg actggggacc tgtaaggaaa cctttaatat gtattacttt gagtcagatg
1081 atcagaatgg gagaaacatc aaggaaaacc aatacatcaa aattgatacc attgctgccg
1141 atgaaagctt tacagaactt gatcttggtg accgtgttat gaaactgaat acagaggtca
1201 gagatgtagg acctctaagc aaaaagggat tttatcttgc ttttcaagat gttggtgctt
1261 gcattgctct ggtttctgtg cgtgtatact ataaaaaatg cccttctgtg gtacgacact
1321 tggctgtctt ccctgacacc atcactggag ctgattcttc ccaattgctc gaagtgtcag
1381 gctcctgtgt caaccattct gtgaccgatg aacctcccaa aatgcactgc agcgccgaag
1441 gggagtggct ggtgcccatc gggaaatgca tgtgcaaggc aggatatgaa gagaaaaatg
1501 gcacctgtca agtgtgcaga cctgggttct tcaaagcctc acctcacatc cagagctgcg
1561 gcaaatgtcc acctcacagt tatacccatg aggaagcttc aacctcttgt gtctgtgaaa
1621 aggattattt caggagagag tctgatccac ccacaatggc atgcacaaga ccccccctctg
1681 ctcctcggaa tgccatctca aatgttaatg aaactagtgt ctttctggaa tggattccgc
1741 ctgctgacac tggtggaagg aaagacgtgt catattatat tgcatgcaag aagtgcaact
1801 cccatgcagg tgtgtgtgag gagtgtggcg gtcatgtcag gtaccttccc cggcaaagcg
1861 gcctgaaaaa cacctctgtc atgatggtgg atctactcgc tcacacaaac tatacctttg
1921 agattgaggc agtgaatgga gtgtccgact tgagcccagg agcccggcag tatgtgtctg
```

FIG. 27 continued

```
1981 taaatgtaac cacaaatcaa gcagctccat ctccagtcac caatgtgaaa aaagggaaaa
2041 ttgcaaaaaa cagcatctct ttgtcttggc aagaaccaga tcgtcccaat ggaatcatcc
2101 tagagtatga aatcaagtat tttgaaaagg accaagagac cagctacacg attatcaaat
2161 ctaaagagac aactattact gcagagggct tgaaaccagc ttcagtttat gtcttccaaa
2221 ttcgagcacg tacagcagca ggctatggtg tcttcagtcg aagatttgag tttgaaacca
2281 ccccagtgtt tgcagcatcc agcgatcaaa gccagattcc tgtaattgct gtgtctgtga
2341 cagtgggagt cattttgttg gcagtggtta tcggcgtcct cctcagtgga agttgctgcg
2401 aatgtggctg tgggagggct tcttccctgt gcgctgttgc ccatccaagc ctaatatggc
2461 ggtgtggcta cagcaaagca aaacaagatc cagaagagga aaagatgcat tttcataatg
2521 ggcacattaa actgccagga gtaagaactt acattgatcc acatacctat gaggatccca
2581 atcaagctgt ccacgaattt gctaaggaga tagaagcatc atgtatcacc attgagagag
2641 ttattggagc aggtgaattt ggtgaagttt gtagtggacg tttgaaacta ccaggaaaaa
2701 gagaattacc tgtggctatc aaaaccctta aagtaggcta tactgaaaag caacgcagag
2761 atttcctagg tgaagcaagt atcatgggac agtttgatca tcctaacatc atccatttag
2821 aaggtgtggt gaccaaaagt aaaccagtga tgatcgtgac agagtatatg gagaatggct
2881 ctttagatac atttttgaag aaaaacgatg ggcagttcac tgtgattcag cttgttggca
2941 tgctgagagg tatctctgca ggaatgaagt acctttctga catgggctat gtgcatagag
3001 atcttgctgc cagaaacatc ttaatcaaca gtaaccttgt gtgcaaagtg tctgactttg
3061 gactttcccg ggtactggaa gatgatcccg aggcagccta caccacaagg ggaggaaaaa
3121 ttccaatcag atggactgcc ccagaagcaa tagctttccg aaagtttact tctgccagtg
3181 atgtctggag ttatggaata gtaatgtggg aagttgtgtc ttatggagag agaccctact
3241 gggagatgac caatcaagat gtgattaaag cggtagagga aggctatcgt ctgccaagcc
3301 ccatggattg tcctgctgct ctctatcagt taatgctgga ttgctggcag aaagagcgaa
3361 atagcaggcc caagtttgat gaaatagtca acatgttgga caagctgata cgtaacccaa
3421 gtagtctgaa gacgctggtt aatgcatcct gcagagtatc taatttattg gcagaacata
3481 gcccactagg atctggggcc tacagatcag taggtgaatg gctagaggca atcaagatgg
3541 gccggtatac agagattttc atggaaaatg gatacagttc aatggacgct gtggctcagg
3601 tgaccttgga ggatttgaga cggcttggag tgactcttgt cggtcaccag aagaagatca
3661 tgaacagcct tcaagaaatg aaggtgcagc tggtaaacgg aatggtgcca ttgtaacttc
3721 atgtaaatgt cgcttcttca agtgaatgat tctgcacttt gtaaacagca ctgagattta
3781 ttttaacaaa aaaaggggga aaagggaaaa cagtgatttc taaaccttag aaaacatttg
3841 cctcagccac agaatttgta atcatggttt tactgaagta tccagttctt agtccttagt
3901 ct
```

FIG. 28 - The published amino acid sequence of EHK1

MRGSGPRGAGRRRPPSGGGDTPITPASLAGCYSAPRRAPLWTCLLLCAALRTLLASPSNEVNLLDSRTVMGDLGWIAFPKNGWEEIGEVDENYAPI
HTYQVCKVMEQNQNNWLLTSWISNEGASRIFIELKFTLRDCNSLPGGLGTCKETFNMYYFESDDQNGRNIKENQYIKIDTIAADESFTELDLGDRV
MKLNTEVRDVGPLSKKGFYLAFQDVGACIALVSVRVYYKKCPSVVRHLAVFPDTITGADSSQLLEVSGSCVNHSVTDEPPKMHCSAEGEWLVPIGK
CMCKAGYEEKNGTCQVCRPGFFKASPHIQSCGKCPPHSYTHEEASTSCVCEKDYFRRESDPPTMACTRPPSAPRNAISNVNETSVFLEWIPPADTG
GRKDVSYYIACKKCNSHAGVCEECGGHVRYLPRQSGLKNTSVMMVDLLAHTNYTFEIEAVNGVSDLSPGARQYVSVNVTTNQAAPSPVTNVKKGKI
AKNSISLSWQEPDRPNGIILEYEIKYFEKDQETSYTIIKSKETTITAEGLKPASVYVFQIRARTAAGYGVFSRRFEFETTPVFAASSDQSQIPVIA
VSVTVGVILLAVVIGVLLSGSCCECGCGRASSLCAVAHPSLIWRCGYSKAKQDPEEEKMHFHNGHIKLPGVRTYIDPHTYEDPNQAVHEFAKEIEA
SCITIERVIGAGEFGEVCSGRLKLPGKRELPVAIKTLKVGYTEKQRRDFLGEASIMGQFDHPNIIHLEGVVTKSKPVMIVTEYMENGSLDTFLKKN
DGQFTVIQLVGMLRGISAGMKYLSDMGYVHRDLAARNILINSNLVCKVSDFGLSRVLEDDPEAAYTTRGGKIPIRWTAPEAIAFRKFTSASDVWSY
GIVMWEVVSYGERPYWEMTNQDVIKAVEEGYRLPSPMDCPAALYQLMLDCWQKERNSRPKFDEIVNMLDKLIRNPSSLKTLVNASCRVSNLLAEHS
PLGSGAYRSVGEWLEAIKMGRYTEIFMENGYSSMDAVAQVTLEDLRRLGVTLVGHQKKIMNSLQEMKVQLVNGMVPL

FIG. 29 - An exon 5' to the published nucleotide sequence of EHK1

TTTCAACCTCGACTTCGCAGCCGCGCACACACCGCCTGCTCCCCGAGCAGCGGGAACCGCAGCAGCTCCTGGCCGCCGCAGCGCAGCTCCGCGCT
CCTACCGGCCGGCAGCCGTCAGTCCCTCCCCTCTTCAGCACTCAGCCCGCAGCTATTTCCTTCTGCCAGTCTCTTTGAACTCTGGATCTTTGCTTT
TGCTCGCTGCTCTCCTGTT

FIG. 30

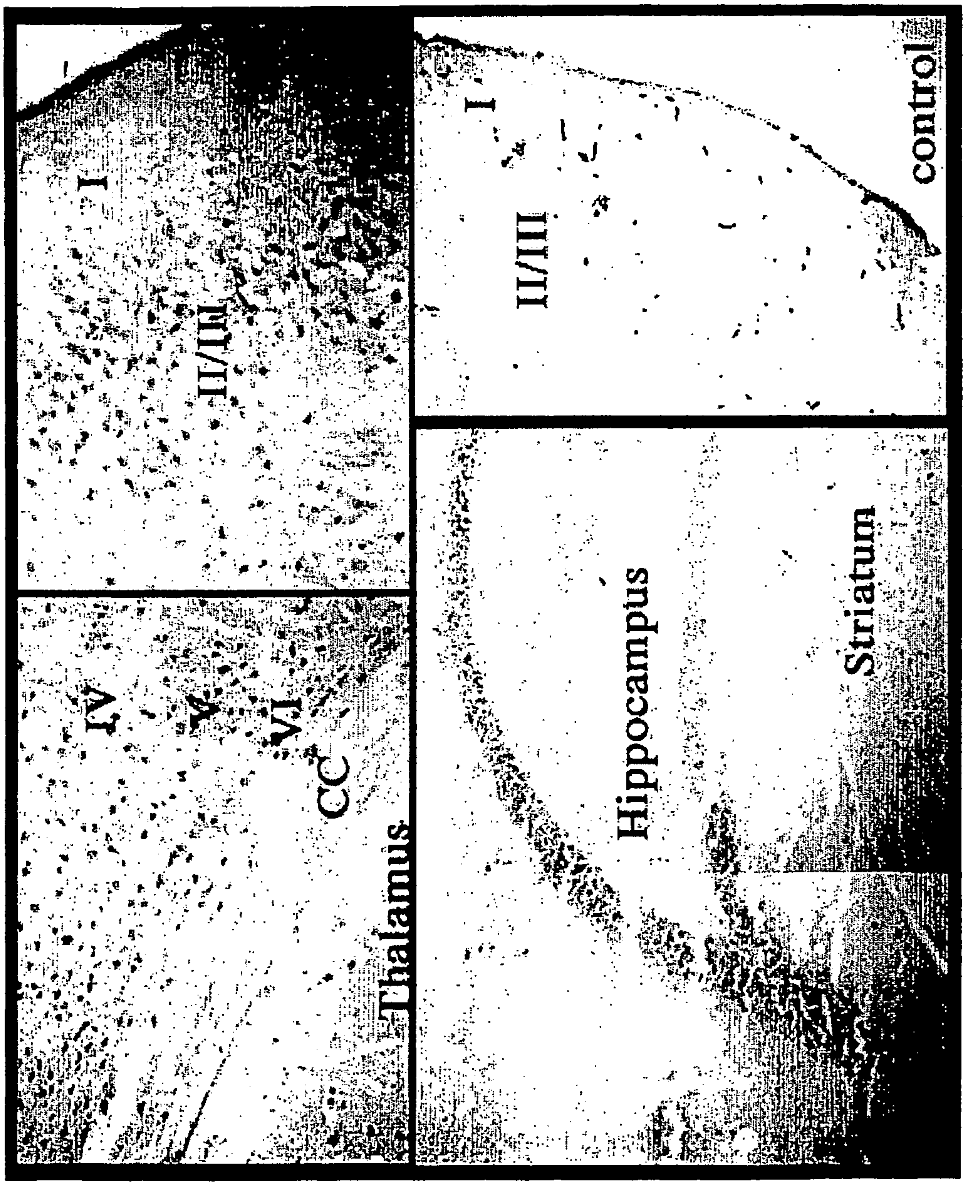

I
II/III
IV
V
VI
CC
Thalamus
control
I
II/III
Hippocampus
Striatum

FIG. 31

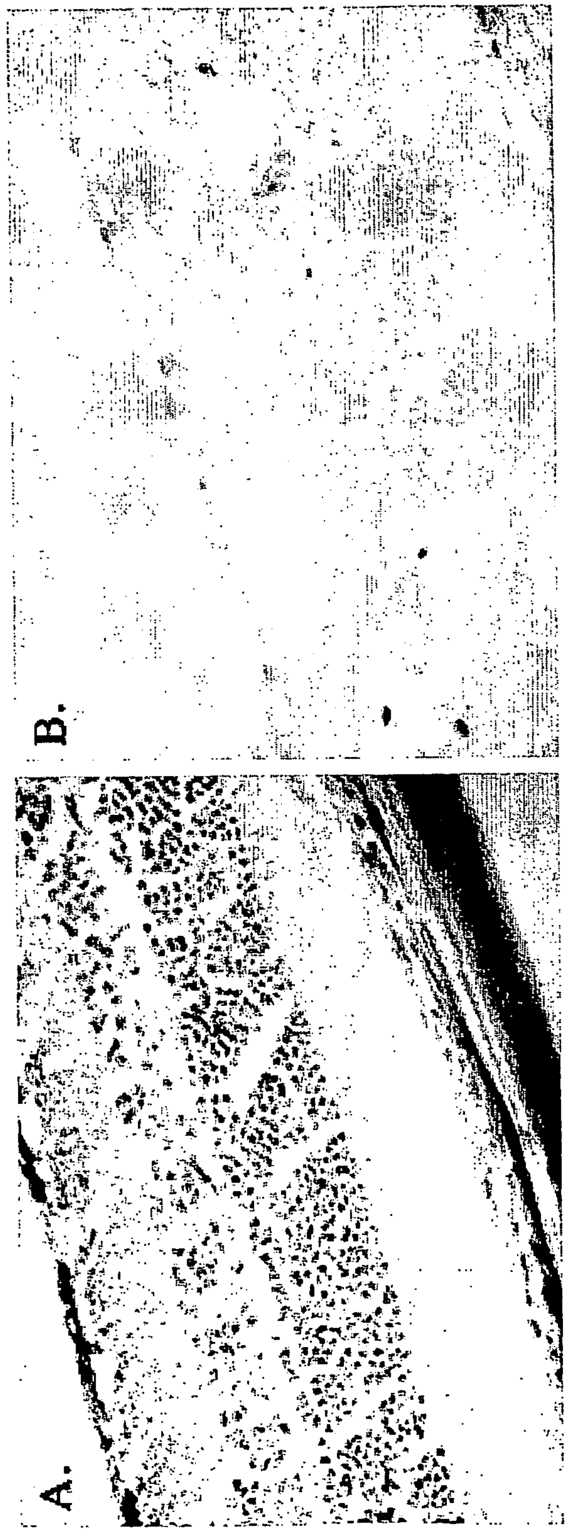
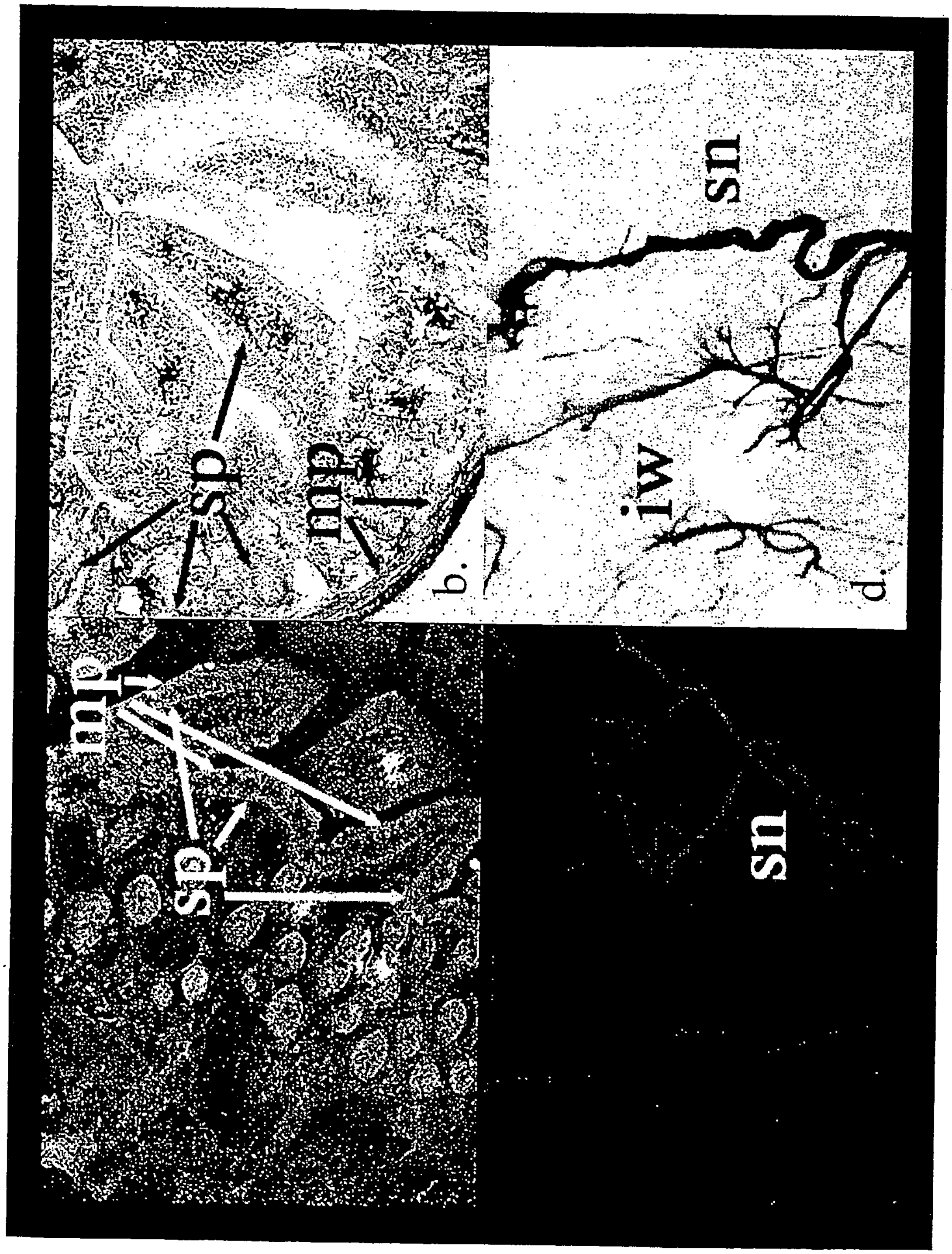


FIG. 32



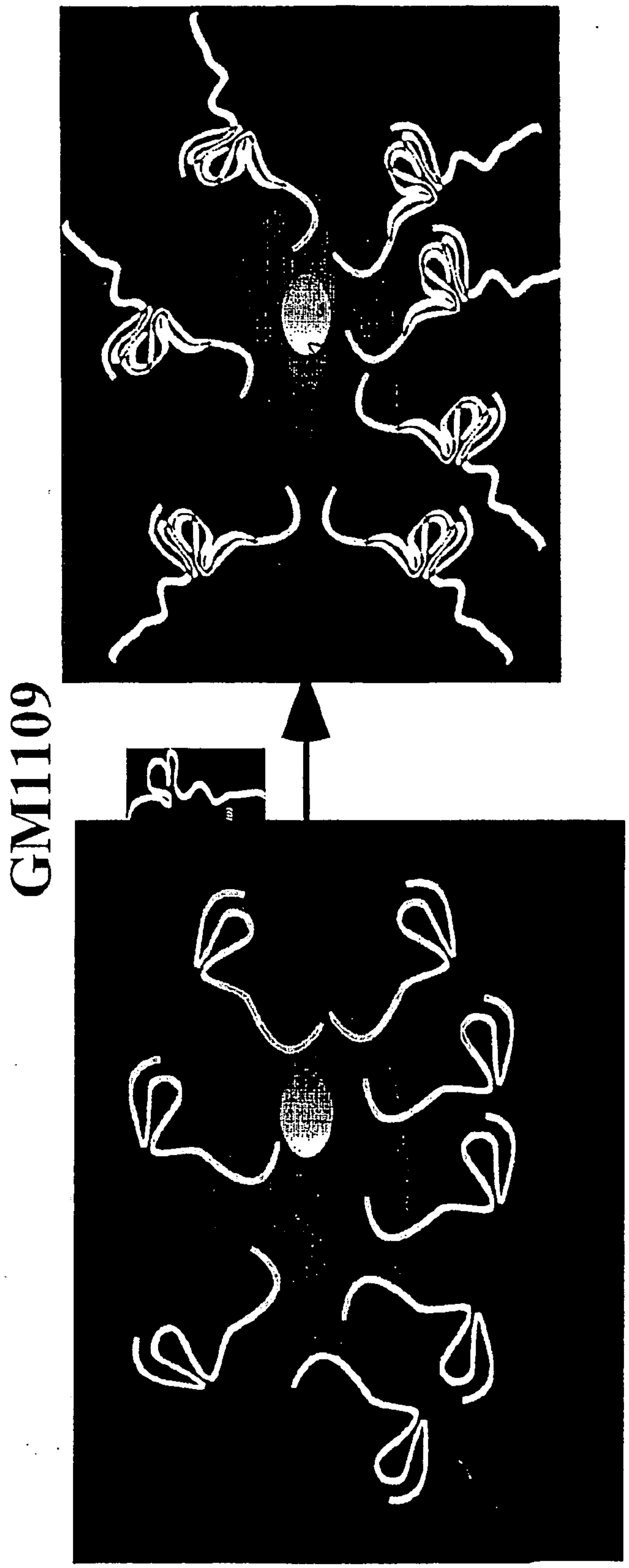


FIG. 33

FIG. 34

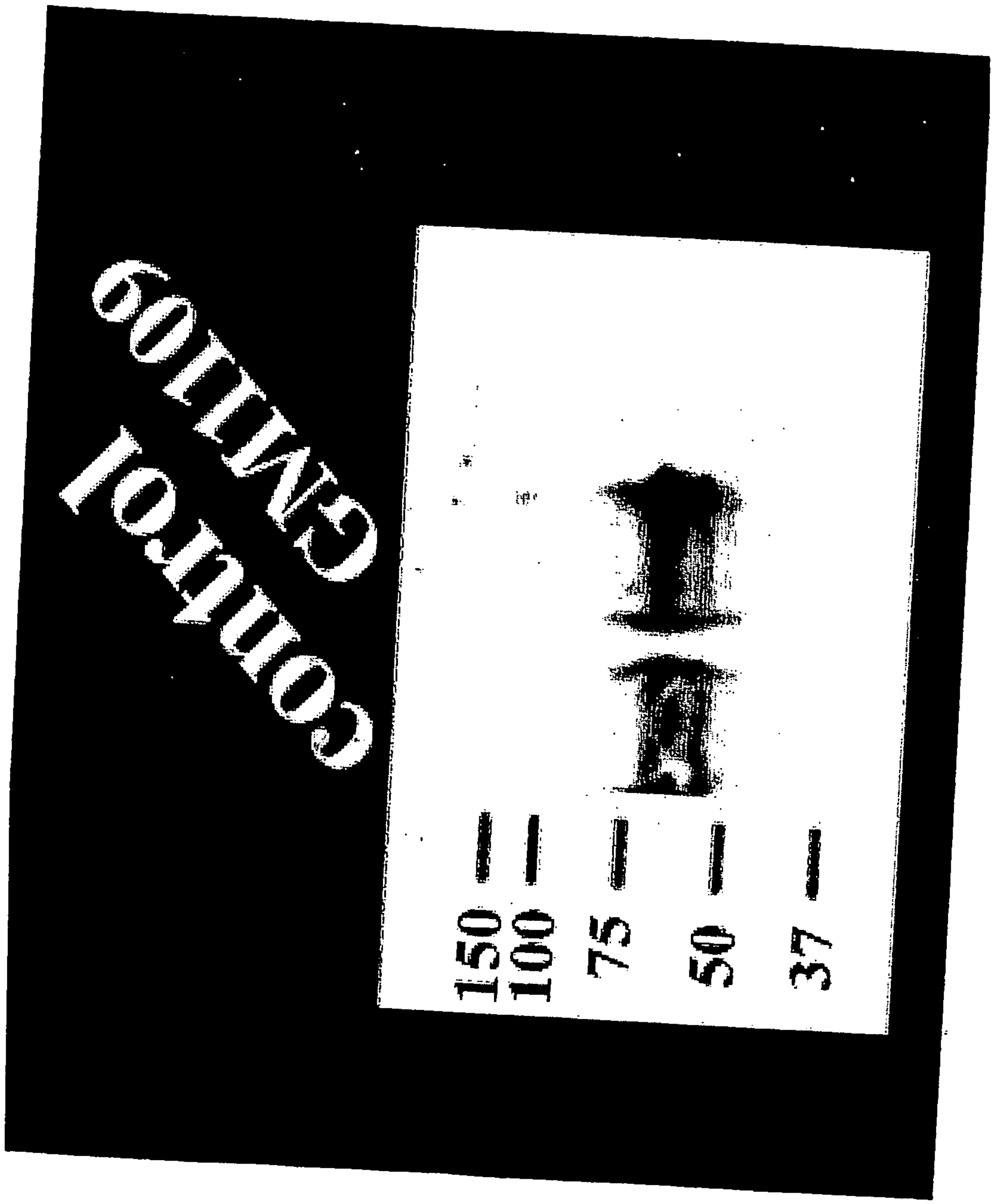


FIG. 35

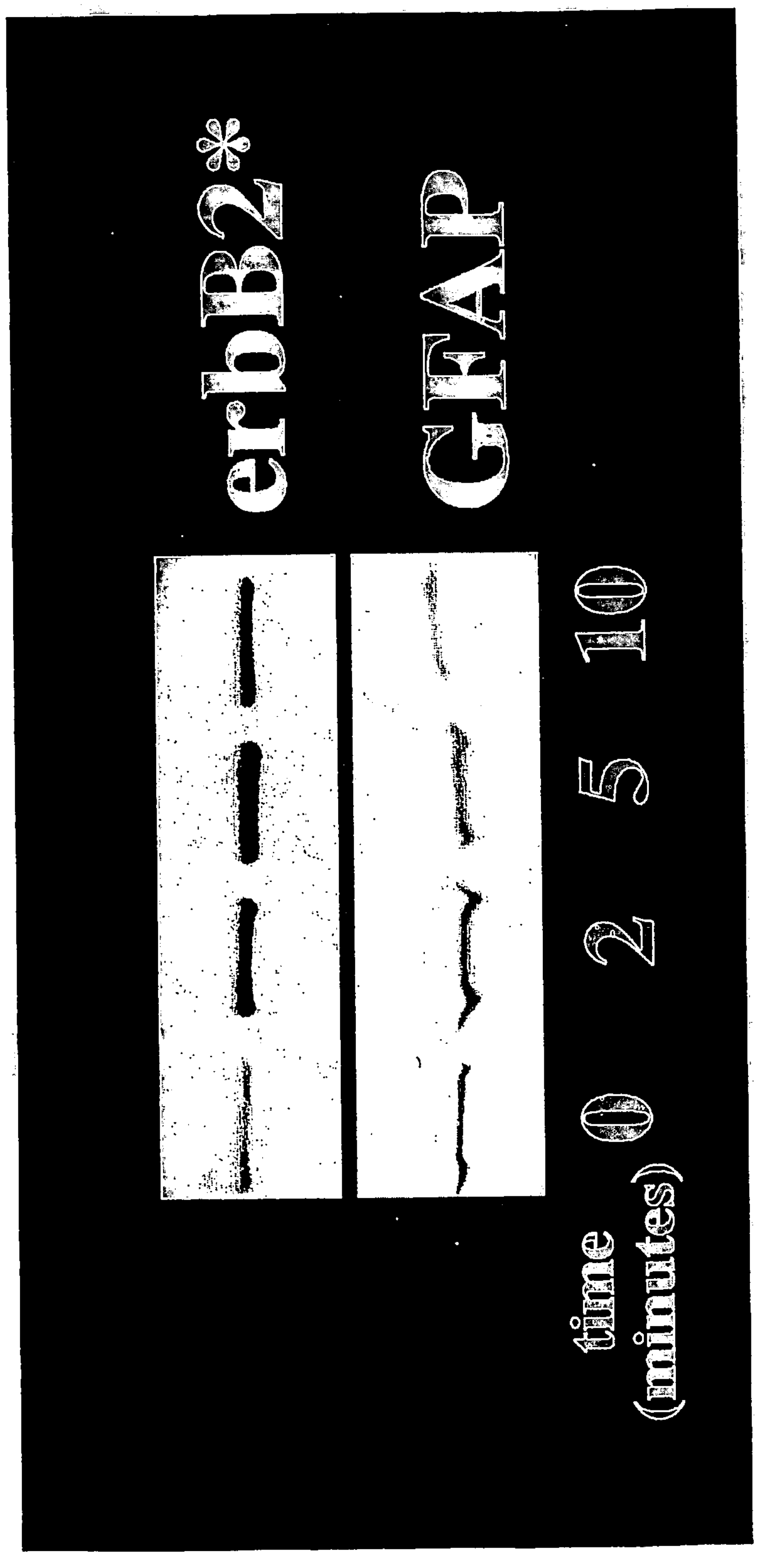


FIG. 36

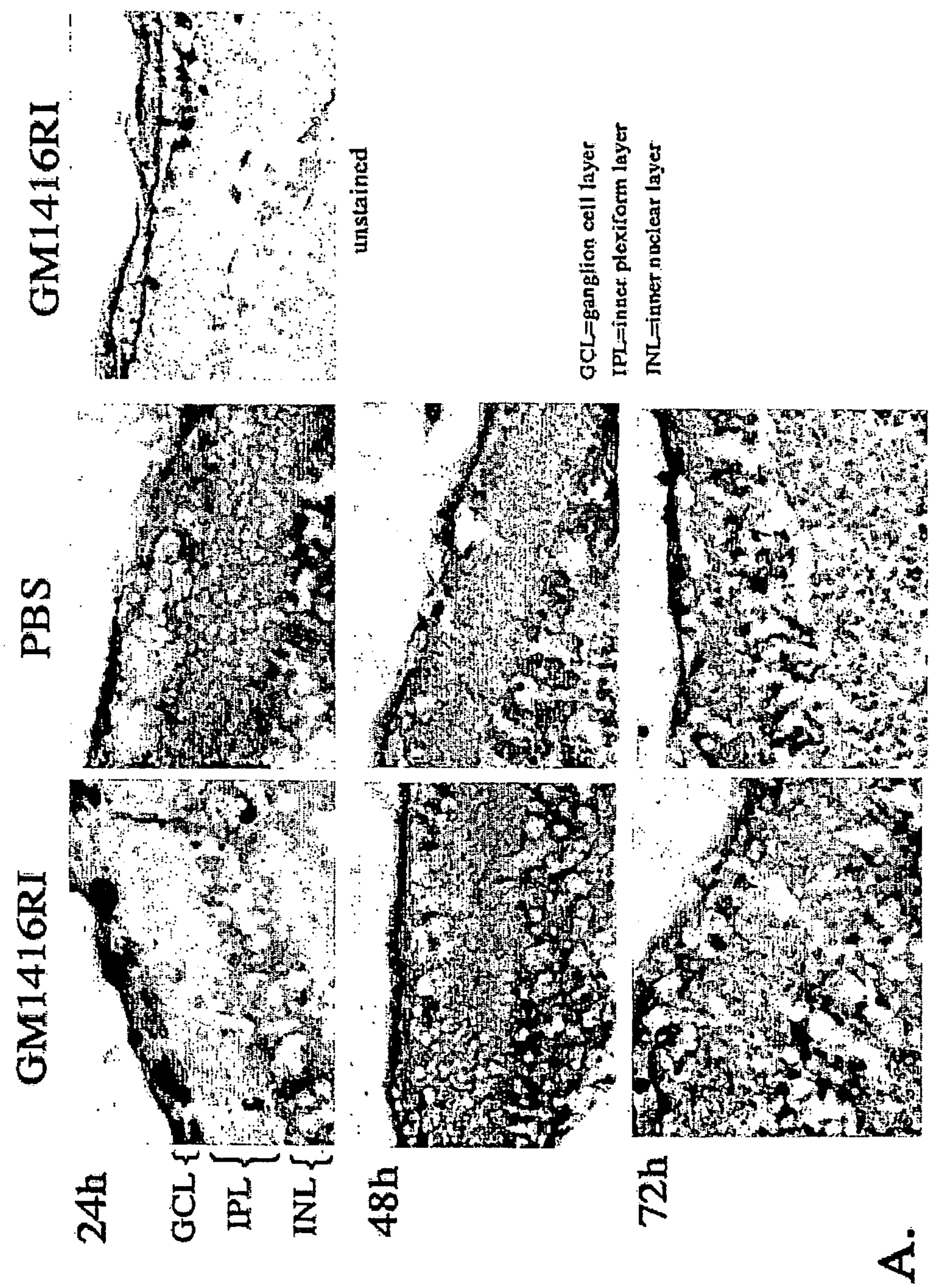

GM1416RI
PBS
GM1416RI
unstained
24h
GCL
IPL
INL
48h
72h
GCL=ganglion cell layer
IPL=inner plexiform layer
INL=inner nuclear layer
A.

FIG.37

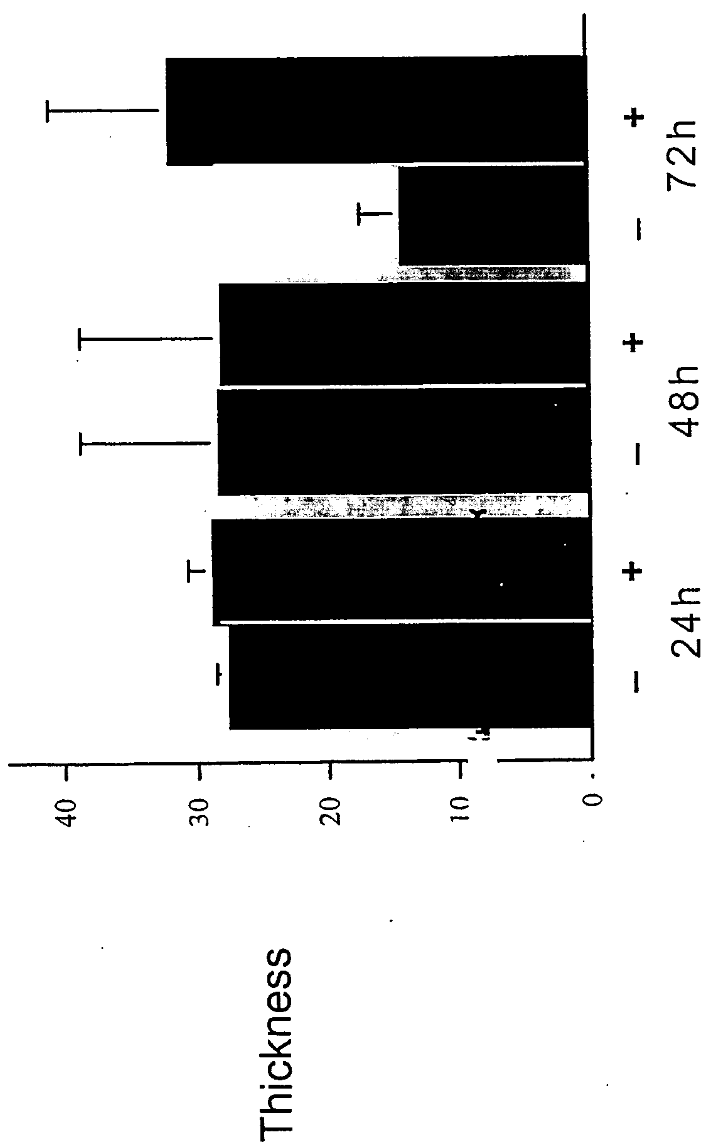


FIG. 38 - Fragments of CD81

A. - GM1109
ETLDCCGSSTLTALTTSVLKNNCCPSGSNIISNLFKEDCHQKIDD

B. - GM1414
ETLDCCGSSTLTALTTSVLK

C. - GM1415
TSVLKNNCCPSGSNIISNLF

D. - GM1416
PSGSNISNLFKEDCHQKIDD

E. - Active 15-mer from GM1416
ISNLFKEDCHQKIDD